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Antimicrobial efficacy of novel titanium dioxide coating and its potential to Induce changes in adaptive resistance

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Abstract

Nosocomial infection is estimated to affect 5% of patients in the United State of America and is likely to be among the top ten causes of early mortality. The first solution that comes to mind is simply hygiene, but the importance of this is often glossed over in favour antibiotics. With rising rates of antibiotic resistance and slowing discovery of new antibiotics, this wellspring is rapidly running dry. Prevention should always be the first option. Improving methods of preventing nosocomial infections is a constant priority. One such method is the use of antimicrobial surface coatings. Metals with antimicrobial agents can be used as self-sterilising coatings.

This thesis focuses on a new formulation of TiO_2 called NsARC. This material has been shown to have photocatalytic properties. Photocatalysis is the use of energy from light (most often UV light) directly to carry out a chemical reaction. This action results in antimicrobial effects because it produces reactive oxygen species (ROS) that destabilise the cell membrane and cell wall. Most often photocatalytic materials are limited to activity in ultraviolet (UV) light, but NsARC shows activity in the visible light spectrum

This thesis first focused first on testing the antibacterial activity of NsARC in UV light, ambient visible light and high intensity visible light. It was found that NsARC has bacterial properties and a proportion of that effect can be attributed to photocatalysis. However, a significant portion of the antibacterial activity was shown to be not light dependant and appears to be caused by a different mechanism.

Previous research has found that some antimicrobial materials can induce higher levels of antibiotic resistance, some herbicides for example. The widespread use of such materials can have a detrimental effect on antibiotic resistance. Given that NsARC is a novel antibacterial

material being proposed for widespread testing of its effects on antibiotic resistance was thought prudent.

This thesis then focused on the design and construction of three reporter strains as indicators of changes in gene expression associated with changes in antibiotic resistance. Suitability of the reporter strains for investigation of NsARC was tested via exposure to herbicides known to induce antibiotic resistance changes. Two of the strains were shown to be suitable for future testing. A preliminary experiment of the effects of NsARC on one of these reporter strains indicated NsARC may induce higher levels of gene expression associated with increased antibiotic resistance. Testing will continue through the use of the reporter strains.

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
ABAC	Active Black Anatase Carbon
ANOVA	Analysis of Variance
ATP	adenosine triphosphate
C	centigrade
CDC	Centres for Disease Control
Cip	ciprofloxacin
Cm	chloramphenicol
DNA	Deoxyribonucleic acid
EOP	Efficiency of Plating
g	grams
HGT	horizontal gene transfer
Kan	kanamycin
L	Litres
LB	Luria broth
MAR	Multi antibiotic resistance
mg	milligrams
MIC	Minimal inhibitory concentration
ml	millilitres
MRSA	Methicillin Resistant *Staphylococcus aureus*
NsARC	Nanostructured Anatase-Rutile Carbon
NZ	New Zealand
ppm	parts per million
ROS	Reactive Oxygen Species
rpm	revolutions per minute
SD	Standard Deviation
Tet	tetracycline
µg	micrograms
µl	microliters
UK	United Kingdom
USA	United States of America

UV	Ultraviolet
WT	wild-type
WHO	World Health Organisation

1.0 Introduction

1.1 Managing Nosocomial Bacterial Infection

Penicillin was the first modern natural products antibiotic, discovered in 1928 (Bérdy, 2012). It saved millions of lives, changing the fates of many people with life threatening infections. Antibiotics quickly became the first choice for dealing with infection. Penicillin resistance is conferred by β-lactamases (Kjellander, Klein, & Finland, 1963). Before the introduction of penicillin as a therapeutic, a β-lactamase was already identified (Abraham & Chain, 1940). Resistance became more common as the use of antibiotics caused a positive selection pressure for resistant bacteria (Kunin, 1993). Different antibiotics were introduced and rapidly became less effective (Kunin, 1993). For example, tetracycline was introduced in 1950 and resistance was observed in 1959. Methicillin was introduced for commercial use in 1960 and resistance was observed as soon as 1962 ("Antibiotic Resistance Threats in the United States, 2013 | Antibiotic/Antimicrobial Resistance | CDC," n.d.). Bérdy, (2012) claims that of pathogenic bacteria, more than 70% are considered resistant to most commercially available antibiotics. A study of isolates of *Staphylococcus aureus* from patients with infections found that 35.9% were clinically resistant to at least one antibiotic in 1992 and this number increased to 64.4% in 2003 (Klevens et al., 2006). In addition, development of novel drugs for use in treatment has reduced significantly (Charles & Grayson, 2004). Antibiotic resistance is biological science's doomsday scenario (L. J. V. Piddock, 2012). In a recent CDC report looking at resistant infections in the United States, it was estimated that resistant strains had resulted in 2 million infections and ~23,000 excess deaths (Michaelidis et al., 2016).

Increasing antibiotic resistance is making this treatment option less effective. In 2002, the estimated number of nosocomial infections in U.S. hospitals was approximately 1.7 million, of these the estimated deaths were 98,987 (Klevens et al., 2007). A more recent study found that the acquisition of a nosocomial infection was associated with doubling the cost of a patient's stay in hospital (Chacko et al., 2017). The situation will only become worse in years to come as antibiotic resistance becomes more prevalent.

Highlighting this problem makes it clear that new antimicrobials are needed, and this is actively driving research. Novel antibiotics seem like a potential solution to antibiotic resistance and this is an area of active research (L. J. Piddock, 2015; Walsh & Wencewicz, 2014), but when new drugs are used, resistance can emerge. A lack of prolonged effectiveness, exacerbated by a lack of enforced stewardship (Amábile-Cuevas, 2016), results in a lower rate of revenue for pharmaceutical companies (Renwick, Brogan, & Mossialos, 2016) therefore less incentive to research novel antibiotics.

Despite all this, the first line of defence against pathogenic antibiotic resistant organisms is preventing spread of these strains and limiting access to immunocompromised individuals. One factor is rigorous hygiene of patients, medical personnel, and equipment to limit pathogens in the environment. Daily washing with a low concentration of chlorhexidine was shown to decrease rates of nosocomial bloodstream infections and the acquisition of multidrug resistant organisms by as much as 23% (Climo et al., 2013). It was shown that after contact with surfaces around a patient one or more pathogen species could be found on the hands of hospital staff more than 50% of the time (Bhalla et al., 2004)

Limiting survival of pathogens on surfaces near these patients can be an effective way of decreasing the risk of infection. On a dry surface, some pathogens can survive for between hours and days. *S. aureus* is one pathogen that can survive months on a dry surface

(Kramer, Schwebke, & Kampf, 2006). The standard for preventing the surface spread of pathogens in hospital scenarios is traditional cleaning with detergents followed by disinfectants (Dancer, 2011). Much of hospital cleaning does not consistently reach current standards of sterility of that country (Griffith, Cooper, Gilmore, Davies, & Lewis, 2000). A pilot study showed that even minor changes in education and less conservative use of disinfectant can reduce the likelihood of finding Methicillin Resistant *S. aureus* from 45% to 27% (Goodman et al., 2008). However, a constant problem with cleaning is that broad flat surfaces are more effectively cleaned than doorknobs, sink or toilet handles (Goodman et al., 2008). Surfaces most often touched are often the hardest to clean and will stay clean for the shortest time, thus becoming a hotspot for touch transmission of pathogens.

An interesting approach to the problem of nosocomial infections has been the use of antimicrobial surface coatings. Some metals are prime candidates for this application.

1.2 Antimicrobial Surfaces

The following section gives an overview of some of the antimicrobial coatings currently in use and being developed. These are copper-based, silver-based, and titanium dioxide.

Copper has been used as an antimicrobial for a long time. The earliest known medical use for copper was described in the Smith Papyrus, written between 2600 and 2200 B.C., where it was used to sterilise chest wounds and drinking water (Dollwet & Sorenson, 1985). However, copper is also an important micronutrient for most living organisms. Some prokaryotes use copper as a co-factor, for example in their cytochrome c oxidase in the electron transport train and superoxide dismutase for minimising oxidative damage (Grass, Rensing, & Solioz, 2011). At higher concentrations copper becomes inhibitory and toxic to microorganisms (Gordon, Howell, & Harwood, 1994). The mechanism by which copper kills

is not fully understood but is thought to be primarily from Cu ions catalysing the production of free radicals in the form of hydroxyl molecules. These hydroxyl molecules then break down the cell walls and membranes, lysing the organism (Grass et al., 2011; Santo et al., 2011). Copper's medicinal properties were one of the main deciding factors for its use in plumbing in the 19th century (Dollwet & Sorenson, 1985). A recent study in a hospital environment compared the rates of aerobic bacteria recovered from copper-coated toilet seats, sink taps, and door push panels with those from their regular stainless-steel versions. Significantly fewer viable bacteria were recovered from the copper surfaces (Casey et al., 2010). This example illustrates that antimicrobial surface coatings may have a positive effect on the survival of pathogens on touched surfaces.

Silver has similar antimicrobial properties to copper and has a similarly long history of use. It has been noted that bacteria often have cell wall and membrane breakdown following silver exposure (Jung et al., 2008). Its historic uses can be traced back to the metallic form being used to make water potable, as people would drop a silver coin in to make it last longer (Alexander, 2009; Klasen, 2000). Moving on from the metallic form, the use of silver compounds in early medicine became more common. More recently the use of silver coated urinary catheters was seen to drop the rate of urinary tract infections from 6.13/1000 catheter days to 2.62/1000 catheter days (Rupp et al., 2004). This resulted in a significant decrease in costs despite the initial cost of the catheters.

There is a resurgent interest in copper, silver and their derivatives for their usefulness as antimicrobial surfaces. Cupric oxide (CuO) and silver oxide (Ag_2O) nanoparticles are also being tested for their photocatalytic properties (Chen et al., 2010; Guo, Hao, Jin, Zhu, & Guo, 2014). Photocatalysis is the use of energy from light (most often UV light) directly to carry out a chemical reaction, in this case water splitting (Ahmad,

Kamarudin, Minggu, & Kassim, 2015). This produces reactive oxygen species (ROS) that destabilise the cell membrane and cell wall. Over time this results in inactivation of the cell, but in the case of CuO surfaces, copper ions can then more easily penetrate the cell and inactivate it quickly (Kazuhito Hashimoto, Irie, & Fujishima, 2005). Silver oxide nanoparticles have been shown to have similar activity, even in the visible light spectrum (Jiang et al., 2015).

A new nano-structured derivative of titanium dioxide (TiO_2 or titania) was the focus of my research. TiO_2 is relatively cheap and abundant (Dalrymple, Stefanakos, Trotz, & Goswami, 2010) and results in a robust surface when used for coating stainless steel or glass (Brook et al., 2007).

The main reason for the recent attention on it is the highly active photocatalytic properties that the coatings display (Foster, Ditta, Varghese, & Steele, 2011). The photocatalytic properties may have both industrial and medical applications. Photocatalytic materials have been highlighted for the possible use in hydrogen production due to the water splitting effect and for its antimicrobial effects. The antimicrobial activity is similar to that of both CuO and Ag_2O. Under UV light, a photocatalytic effect produces ROS that destabilise membranes (Block, Seng, & Goswami, 1997). The killing mechanism involves breakdown of the membrane/cell wall because of ROS such as hydroxyl radicals and hydrogen peroxide formation. This causes leakage of cellular contents, then cell lysis that may be followed by complete mineralisation of the organism if the coating is highly active (Dalrymple et al., 2010).

The cost of both copper and silver is limiting, titania is less expensive. Just a cursory inspection of international metal prices shows that in January 2018 copper was worth 6.79 USD/Kg, silver is 591.47 USD/Kg and titanium is only 4.90 USD/Kg (“Metal Price Charts -

InvestmentMine" 2018.). One of the limitations of photocatalysis by titania is that many present formulations are most active only when excited by UV light. Previous research has been done on making novel titania coatings that are more active in the visible spectrum. Although this process has produced coatings that were more active than comparators in visible light, their overall activity was still far lower than in UV light (Asahi, Morikawa, Ohwaki, Aoki, & Taga, 2001; Q. Li, Xie, Li, Mintz, & Shang, 2007; Wong et al., 2006). Given the limited success with visible light, there is a great deal of interest for a new formulation that is currently being developed by a group within the University of Canterbury School of Engineering. The novel form is called Active Black Anatase Carbon (ABAC) or Nanostructured Rutile, Anatase and Carbon (NsARC).

The new formulation of TiO_2 is made as a thin film, deposited by a pulsed-pressure Metal-Organic Vapour Deposition (pp-MOCVD) process, which uses flash vaporization of liquid precursor, causing a sharp spike in vapor pressure (D. Lee, Krumdieck, & Talwar, 2013). This process creates a novel TiO_2 material, a composite of nanostructured anatase, rutile and carbon, which has a surface area two orders of magnitude greater than that of an uncoated surface. Preliminary tests have shown the potential for significant photocatalytic activity in both UV and visible light.

Prior to this thesis, testing was required to ascertain the effectiveness of the different formulae for producing redox species from water, and the overall effectiveness as an antimicrobial coating. To accomplish that, methylene blue was used as a chemical indicator. Methylene blue (MB+) is a dye commonly used as a surrogate measure of antimicrobial ROS generation by new photocatalytic materials (Mills, Hill, & Robertson, 2012). Methylene Blue ($C_{16}H_{18}ClN_3S$) is blue in solution, but when oxidised in solution it is bleached and then mineralised (Mills, Davies, & Worsley, 1993). Spectrophotometry can be

used to measure the rate of bleaching. When methylene blue is exposed to a photocatalytic surface, the rate of bleaching can be used to measure the rate of oxidation from the photocatalytic surface. This is how photocatalytic activity was initially tested for NsARC.

1.3 Antibiotic resistance on the rise

With proper education, the decades of overuse of antibiotics are slowly coming to an end. As with antibiotics, antimicrobial coatings may select for pathogenic bacteria that are resistant to killing. Indeed, there are reports of both an increase in resistance and associated virulence for some bacteria after surviving exposure to ROS (Tsai Ting-Mi, Chang Hsin-Hou, Chang Kia-Chih, Liu Yu-Lin, & Tseng Chun-Chieh, 2010).

Bacteria are resistant to antibiotics through inactivation of the antibiotics, keeping the antibiotic concentration low in the cell, or changing the target site of the antibiotic (J. Lin et al., 2015).

Inactivation of the antibiotic molecule occurs via enzymes degrading or making molecular changes to it (Davies, 1994). The first clinical antibiotic resistance mechanism discovered was β-lactamases that deactivate the β-lactam ring of penicillin and derivatives (Majiduddin, Materon, & Palzkill, 2002).

Evolutionary pressure can result in the target of the antibiotic molecule being changed so that the antibiotic molecule cannot bind. Mutation in the *gyrA* and *parC* genes that change DNA maintenance proteins to provide high level resistance to fluoroquinolones is an example of this (Janoir, Zeller, Kitzis, Moreau, & Gutmann, 1996).

Antibiotics can also be actively removed from the cell interior by active efflux transport pumps. Efflux pumps in *Escherichia coli* like AcrEF-TolC are associated with

fluoroquinolone resistance, while AcrAB-TolC can export multiple different types of antibiotics (Webber & Piddock, 2003). There are several different families of multidrug efflux pumps, some of these include the resistance-nodulation-cell division (RND), major facilitator (MF), staphylococcal/small multidrug resistance (SMR), multidrug and toxic compound extrusion (MATE) families and ATP-binding cassette (ABC) (X.-Z. Li & Nikaido, 2004). Efflux by proteins in the first five families is driven by secondary transport, being moved down the electrochemical gradient by the co-movement of protons and sodium ions. Efflux in the ATP-binding cassette transporters is powered by ATP hydrolysis. Efflux systems come in two general types. Some, like the tetracycline resistance pump (TetA) (Schnappinger & Hillen, 1996) are a single component efflux system with a high degree of substrate specificity, acting on a few agents or multiple agents within the same drug class. Others form protein complexes and are able to confer resistances to multiple antibiotics, the RND family is an example of these (Blair & Piddock, 2009).

Another way of categorising antibiotic resistance is via the origin of the resistance phenotype: how the bacteria came to have a given mechanism. Acquired resistance is a change in the genotype and can arise within a population via gene acquisition or mutation, this can be seen in the resistance mechanism of β-lactamases altering the β-lactam ring of penicillin and derivatives as they are often spread within populations via horizontal gene exchange (Majiduddin et al., 2002). Intrinsic resistance is due to the genotype of the organism and is uniform in the species (Cox & Wright, 2013), this can be seen the resistance of *Proteus* to polymyxins (Olaitan, Morand, & Rolain, 2014). Adaptive resistance is similar to intrinsic resistance, but it is environmentally induced, usually by transcriptional activation. The genes *tolC, marR,* and *soxS* are part of systems associated

with efflux and adaptive resistance.

The AcrAB-TolC protein complex is part of the RND protein family. It is now one of the best understood efflux pathways and is associated with efflux of the widest range of antimicrobial agents (Baucheron et al., 2004; Motta, Cluzel, & Aldana, 2015). This pump assembly comprises the outer-membrane channel TolC, the secondary transporter AcrB located in the inner membrane, and the periplasmic AcrA which lies between membranes and connects between TolC and AcrB. The AcrAB-TolC efflux pump can transport compounds with little chemical similarity, thus conferring resistance to a broad spectrum of antibiotics (Du et al., 2014). The AcrAB-TolC efflux pump system has been found to be important for *E. coli* to resist many antibiotics including tetracycline, chloramphenicol, fluoroquinolones, rifampicin and β-lactams (Li & Nikaido, 2004; Nikaido, 1996). The AcrAB-TolC efflux system and many others like it are under tight control by transcriptional regulators.

The multiple antibiotic resistance (*marRAB*) operon in *E. coli* was discovered during studies of tetracycline resistance and is used as a model of changes in gene expression causing resistance to antibiotics (Prajapat, Jain, & Saini, 2015). The *marRAB* operon controlled via positive and negative feedback by the proteins MarA and MarR. These protein are DNA-binding transcriptional regulatory proteins (Prajapat et al., 2015). When lacking any stress signals MarR binds to the *marRAB* promoter and represses gene expression (Martin & Rosner, 1995). When inducers like salicylate and other phenolic compounds are present, MarR binds to them and is then unable to bind to the *marRAB* promoter (Cohen, Levy, Foulds, & Rosner, 1993). And in this case MarA acts as an activator of the system when bound to the *marRAB* promoter.

The target genes of MarA are regulated positively or negatively and control physiology in several ways that tend to increase cell survivability when subjected to stress (Prajapat et al., 2015). The effects on the genes targeted by MarA include upregulation of efflux pumps, including *acrAB* and *tolC*, and downregulation of the porin gene *ompF* which decreases influx.

SoxS is another transcription factor and a homologue of MarA (Storz & Imlayt, 1999). SoxS has been found to be vital for the regulation of many proteins that play roles in defending *E. coli* from oxidative stress including superoxide dismutase (SOD), DNA repair endonuclease IV, glucose 6-phostphate dehydrogenase (Nunoshiba, Hidalgo, Cuevas, & Demple, 1992) and the AcrAB efflux system (Gu & Imlay, 2011). It is also been shown that its induction with an inducible promoter mutant increases antibiotic resistance (Greenberg, Monach, Chou, Josephy, & Demple, 1990). Past research has shown that that SoxR seems to act as a redox sensor and is induced by superoxide (O_2^-), often generated by chemical attack on the cell (Zheng, Doan, Schneider, & Storz, 1999). This induction causes SoxR to bind to the *soxS* promoter and induce transcription in genes associated with redox defence (Storz & Imlayt, 1999). More recent research shows evidence to support the theory that SoxR is not induced by superoxide, but by "redox-cycling drugs" (e.g. paraquat or menadione). This was shown by exposing *E. coli* to excess superoxide and it not effectively activating SoxR in a superoxide dismutase deficient (SOD^-) mutant and an overexpressing SOD mutant not being able to suppress SoxR activation by "redox-cycling drugs" (Gu & Imlay, 2011).

The changes of antibiotic resistance induced by non-antibiotic antimicrobials are becoming more closely studied in recent years. Some of the changes in antibiotic resistance caused by non-antibiotic antimicrobials has been attributed to changes in expression of genes associated with efflux (Kurenbach et al., 2015). Changes in antibiotic resistance

caused by herbicides have been shown in recent research from multiple commercial formulations of herbicides. The herbicides tested include Kamba500, which contains the active ingredient dicamba, 2,4-D amine 800 WSG, which contains 2,4-dichlorophenoxyacetic acid (2,4-D), and Roundup weed killer, which has the active ingredient glyphosate (Kurenbach et al., 2015). This research showed that it is not just the irresponsible use of antibiotics that is causing antibiotic resistance, but potentially the excessive use of many antimicrobial products. Copper (II) sulphate is also a commonly used herbicide, but research has shown that Cu^{2+} may be the key inducer of MarR and thus cause changes in antibiotic resistance (Hao et al., 2014). Copper has recently been associated with the potential induction of tetracycline resistance (Jun, 2017). Because of these examples I believe that it is necessary to fully test the possible effects on antibiotic resistance of any new antimicrobial materials that may be introduced. The novel form of titania that is the focus of this research is no exception. My thesis is about a novel surface coating hypothesised to have antimicrobial properties. Based on its composition and the structure of the material at the nano-scale, the antimicrobial activity is the result of photocatalytic generation of reactive oxygen species.

Much of the previous literature has outlined the effectiveness of titania within the context of water purification using nanoparticular forms (Caballero, Whitehead, Allen, & Verran, 2009; Li et al., 2007), so more research is needed on the effectiveness of these substances as solid coatings. Antimicrobial testing on other photocatalytic substances has included testing on model bacteria like *E. coli* (Caballero et al., 2009). Other research has included analogues for human pathogens, such as different phages and fungi (Cho, Chung, Choi, & Yoon, 2005; Erkan, Bakir, & Karakas, 2006; Gerrity, Ryu, Crittenden, & Abbaszadegan, 2008; Lee, Nakamura, & Ohgaki, 1998; Maneerat & Hayata, 2006). They have

all found a significant killing effect from photocatalytic activity, but it has largely been limited by UV light activation. Success with visible light antimicrobial photocatalysis has been limited.

I could find little research on the potential effects of photocatalytic antimicrobial activity on antibiotic resistance. This would be highly relevant to nosocomial infection transmission, incomplete sterilising or nonlethal exposure to the material could result in changes in antibiotic resistance, possibly due to triggering adaptive resistance. This is relevant for the efflux pumps and transcription regulators mentioned above, especially the *soxRS* locus, thought to be induced by redox activity which photocatalytic activity would certainly generate.

1.4 Objectives

The aim of this thesis is to first investigate whether NsARC has antimicrobial properties. It aims to investigate the extent of any antibacterial activity and whether or not this activity can be attributed to photocatalytic activity. Furthermore, it aims to test whether NsARC is an antimicrobial substance that may induce higher levels of antibiotic resistance by causing changes in expression of efflux genes.

To this end I tested the antibacterial effects of NsARC under UVA light (315–400 nm) and visible light (450-650 nm) and compared them with activity in darkness. I used *E. coli* as a model organism for the photocatalytic antimicrobial activity of NsARC.

Secondly adaptive resistance due to changes in efflux pump expression in *E. coli* has been well studied in relation to exposure to herbicides (P. S. Gibson, 2016; Kurenbach et al., 2015). The expression of the genes *tolC, marR,* and *soxR* was the subject of these experiments, also because of their familiarity within the lab group. Their promoter regions

were made part of gene constructs. Within each construct one of the promoter regions controlled expression of a fluorescent protein. The strains containing these constructs were subjected to initial testing against herbicides already associated with inducing an antibiotic resistant state (Kurenbach et al., 2015). This was to be followed by testing of the reporter strains against NsARC and inert control surfaces.

I started with two hypotheses.

Hypotheses

1. NsARC antimicrobial activity is due to photocatalysis
    a. NsARC has significantly greater killing effect illuminated by UVA light than inert surfaces or NsARC in the dark against *E. coli.*
    b. NsARC has significantly greater killing effect illuminated by visible light than inert surfaces or NsARC in the dark against *E. coli*
2. Exposure to NSARC antimicrobial will cause increased expression of efflux control genes associated with antibiotic resistance
    a. *E. coli* containing a gene construct with an efflux control gene promoter controlling the expression of a fluorescent protein will have significantly higher brightness levels when exposed to NSARC than inert surfaces.

2.0 Methods

2.1 Experimental Methods

2.1.1 Bacterial Strains, culture conditions and chemicals

Strains of *E. coli* used in this study are shown in Table 2.1. The *E. coli* strain that I used for antibacterial testing was ATCC® 8739™, a Crooks strain with a long history of use as a type strain (Gunsalus & Hand, 1941). This strain was selected due to its common usage in photocatalytic research and is one of the suggested strains used in ISO for photocatalytic surfaces (Foster et al., 2010). The type strain of *E. coli* used in the reporter strains was BW25113 (Grenier, Matteau, Baby, & Rodrigue, 2014), given the in-depth understanding of its responses to many antimicrobials close at hand, it is the best choice for a model organism for gene expression changes.

In all cases bacterial strains were stored long term at -80°C in glycerol solution (15%). When revived, they were maintained at 4°C but refreshed from the frozen stocks every week. Cultures were maintained for no more than one week at a time on Nutrient broth (NB) Agar (Bacteriological Agar No.1, Oxoid (UK)) at 4°C and grown in (NB) at 37°C in a gyratory water bath shaker for aeration. Cultures were maintained for no more than one a week at a time on Luria-Bertani (LB) Agar at 4ºC and grown in LB broth at 37°C in a gyratory water bath shaker for aeration prior to experiments. Luria-Bertani base (Lennox-L-Broth Base, Invitrogen (USA)) and agar (Bacteriological Agar No.1, Oxoid (UK)) were used.

Table 2. 1: Bacterial strains and plasmids used in this study.

	Genotype	Reference
E. coli		
ATCC8739	"Crooks Strain"	(ISO, 2009)
BW25113	F^-, λ^-, Δ*(araD-araB)567*, *ΔlacZ4787(::rrnB-3), rph-1,*Δ*(rhaD-rhaB)568, hsdR514*	(Baba et al., 2006)
Plasmids		
pTolC-mScarlet	*cat, nptII, pBBR1, tolC promoter:mScarlet-I*	This study
pMarR-mScarlet	*cat, nptII, pBBR1, marR promoter:mScarlet-I*	This study
pSoxS-mScarlet	*cat, nptII, pBBR1, soxS promoter:mScarlet-I*	This Study
E. coli with plasmids		
BWtolC	BW25113 (pTolC-mScarlet*)*	This study
BWmarR	BW25113 (pMarR-mScarlet*)*	This study
BWsoxS	BW25113 (pSoxS-mScarlet*)*	This study

The antibiotic used in these experiments was kanamycin (stock concentration: 40 mg/ml, Life Technologies (USA)

Kamba 500 (Nufarm, NZ) was obtained from PGG Wrightson in a liquid form. Kamba 500 contains the active ingredient dicamba as a dimethylamine salt (500 g/L) and was kept in a stock concentration (2260 mMol) at room temperature. It was exclusively used at the concentration 1.95 g/L, well below the MIC of Kamba 500 (Kurenbach et al., 2015). 2,4-dichlorophenoxyacetic acid (2,4-D) (sodium salt monohydrate) was purchased from Sigma-Aldrich (USA). 2,4-D was dissolved in double-distilled, autoclaved water to the concentration of 300 mMol for stock solution and stored at room temperature. It was exclusively used at the concentration of 1.95 g/L. A fungicide, Liquid Copper (Yates, Auckland, NZ) was purchased commercially. The active ingredient in this product was copper (92.8g/L) (Cu) present as copper ammonium acetate. This was used exclusively at a sub-lethal concentration (Jun, 2017) of 4.73 g/L.

Paraformalderhyde was used for fixation of cells prior to fluorescence microscopy. It was obtained from Sigma-Aldrich (USA) was kept as powdered stock at 4^{o}C. 4% solution of paraformaldehyde was made up on the day it was to be used and kept at 4^{o}C. Any remainder was discarded after use.

Gelatine coated slides were made by washing glass microscopy slides (Mareinfeld-Superior, 76x 26 mm, approx. 1 mm thickness) in 70% ethanol for 1 hour. They were then airdried and dipped in 0.1% gelatine solution at 70^{o}C and then airdried. After drying they were kept at 4^{o}C and disposed of after 1 week if not used.

2.1.2 Coated and uncoated surfaces supplied for testing

Initially Koti Technologies and the lab group of Professor Susan Krumdieck kindly supplied 50 mm x 50 mm pieces of test material. The initial supply consisted of 36 pieces of 304 stainless steel coated with depositions of NsARC. We were informed that 14 pieces of these appeared to be of good functioning quality, the set having been sampled and showing activity using methylene blue standardised testing methods (Mills et al., 2012). The other 18 were of uncertain activity levels. We were informed they displayed little to no photocatalytic activity with methylene blue, because of this these 18 were not used in testing. The initial supply of only 14 samples severely limited initial experiments, despite assurances that they were reusable. We were also supplied with 9 uncoated pieces of 304 stainless steel and 6 pieces of aluminium, also 50 mm x 50 mm for use as negative controls.

Later I received fresh depositions of NsARC samples. Newer sets of samples were 25 mm x 25 mm pieces of coated 304 stainless steel. Negative controls for these samples were glass microscopy slides (Mareinfeld-Superior, 76x 26 mm, approx. 1 mm thickness) cut to approximately 25 mm x 25 mm size.

NsARC samples and controls (stainless steel/aluminium/glass) were kept in the dark for 24 hours prior to any experimental use to ensure full discharge of any photoactivity.

2.1.4 Testing antibacterial properties in Ultraviolet light

The experiment is based on BS ISO 27447: Test method for antibacterial activity of semiconducting photocatalytic materials (H. A. Foster et al., 2010). NB was inoculated with a single colony of *E. coli* ATCC8739. This was incubated at 37°C with agitation for aeration until saturation. This culture was serially diluted and the dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were spot plated as 10 µl drops in triplicate as shown in (figure 2.1) on a nutrient agar plate.

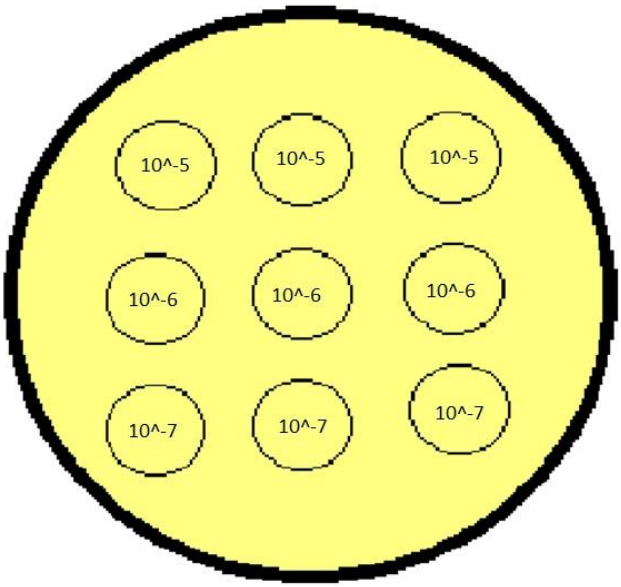


Figure 2. 1: 90 mm agar plate. Each circle represents 10 µl dropped on the plate in a spot. Within each circle is the corresponding dilution factor of the 10 µl spot on the plate.

Starting culture for this experiment was diluted to approximately $1x10^7$ cfu/ml. Test pieces were then placed in sterile petri dishes on sterile glass slides which were separating the samples from wet sterile filter paper at the bottom of the petri dish (Figure 2.2).

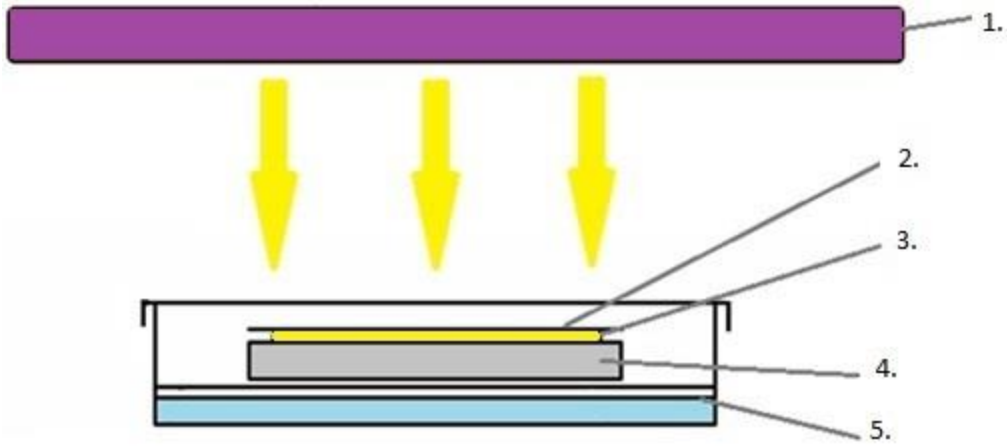


Figure 2. 2: Experimental set up for testing antibacterial activity of surfaces under different light exposures. 1: UV light with a peak wavelength of 365 nm. 2: 24x24 mm microscope coverslip. 3: bacterial culture of 50 µl containing approximately 500000 cfu. 4: material being tested for antibacterial activity, either NsARC (25x25 mm) or glass (25x25 mm). 5: filter paper dampened with sterile distilled water to preserve moisture. Items 2-5 are within a 90 mm plastic petri dish to limit contamination and preserve moisture.

Approximately $5x10^5$ bacteria (50 µl of culture) were placed on samples and covered with microscope cover slips (24x24 mm) to spread culture across sample.

Treatments of time were either 0 or 4 hours. Treatments of surface were either NsARC or glass. Treatments of light variation were either light or dark. Light treatment refers to a UV lightbox supplied by Koti Technologies. The fluorescence bulbs produced a light at a peak wavelength of 365 nm.

Samples were washed off after treatments using 1.95 ml of Tryptic Soy Broth with 0.05% Tween 80. Various dilutions were plated on Tryptic Soy Agar. Three dilutions were then spot plated in 3 replicates per dilution. With 10µl in each spot on a plate, dilutions were as shown in Figure 2.1. Plates were incubated at 30°C for 18 hours and colonies counted. The number of colony forming units (cfu) of the bacteria between the two treatment groups was compared. Because there was a variability in the concentration of *E. coli* between saturated cultures, the cfus were normalized to efficiency of plating (EOP). The

$$EOP = \frac{CFU\ in\ treatment\ plate}{CFU\ in\ No\ treatment\ plate}$$

Figure 2. 3: Equation for calculating Efficiency of Plating

formula for EOP is shown in figure 2.3. Each experiment was carried out three times independently.

2.1.5 Testing antibacterial properties in ambient visible light

This experiment was performed as in section 2.1.4, but with ambient light of 650-750 lux found within the lab in the windowless room illuminated by fluorescent bulb overheard lighting (450-650 nm) in Light treatments.

2.1.6 Testing antibacterial properties in high intensity visible light

This experiment was performed as in 2.1.4, but with a light box to illuminate samples in Light treatments. This lightbox produced a light intensity of approximately 2100 lux (450-650 nm).

2.1.7 Investigating fluorescent response of reporter strains using copper ammonium acetate

Methods for construction of the reporter strains can be found in Appendix A. Prior to section 2.1.7.1 the reporter strains were tested for plasmid stability (appendix B) and growth rates were observed with and without copper ammonium acetate exposure (appendix C).

2.1.7.1.1 Exposure of BWtolC and BWsoxS strains to copper ammonium acetate

Each strain was grown to saturation (approximately 1 x 10^9 cfu/ml) then serially diluted. The dilutions were spot plated to ascertain exact concentration as shown in section 2.1.4 (Figure 2.1). The following was performed in triplicate for each strain. 100 µl of 1 x 10^7 cfu/ml saturated culture was put in an Erlenmeyer flask with 10 ml of LB. 100 µl of 1 x 10^7 cfu/ml was also put in an Erlenmeyer flask with 10 ml of LB and copper ammonium acetate (4.73 g/L). Both flasks were incubated in a water bath shaker at 37^{o}C at 215 rpm for 140 minutes.

Immediately following incubation, the cells were subjected to paraformaldehyde fixation (see section 2.1.6.2)

2.1.7.2 Paraformaldehyde fixation of cells

Each treatment of cells fixed via paraformaldehyde was treated separately as follows. Cells were pelleted for 1 min. The supernatant was discarded. The pellet was resuspended in 50-100 µl 1x Phosphate Buffer Saline (PBS). 2 volumes of 4% Paraformaldehyde fixing solution was added to the tube. The tube was stored for 1-3 hours at 4^{o}C. The ells were pelleted by centrifugation for 1 min at approximately 6500 xg. The supernatant was discard. The cells were washed one time with at least 4 volumes of 1xPBS. Refilled with appropriate volume of 1xPBS. Stored at -20 C in 50% ethanol.

2.1.7.3 Mounting of fixed cells on gel coated cells

2 µl of fixed cells from 2.1.7.3 was spread in a circular smear on a gel-coated glass slide and allowed to dry at room temperature. 6 µl of 50% glycerol was then pipetted on top of the dried smear and spread out underneath a 25 x 25 mm glass cover slip.

2.1.7.4 Fluorescence Microscopy Imaging on BWtolC and BWsoxS

Fixed and mounted cells were examined with an Axio Imager.M1 (Zeiss, Oberkochen, Germany) using 556/20 nm excitation bandpass. Digital images were captured at 1000x magnification with an AxioCam MRm camera (Zeiss) in differential interference contrast (DIC) and through a 556/20 nm (red) filter set. Approximately 30 images were taken for each treatment replicate.

2.1.7.5 Image Processing and Analysis for BWtolC and BWsoxS strains with copper ammonium acetate

Images were analysed using Fiji ImageJ (Schindelin et al., 2012). The images were converted to 8-bit to speed processing. Each fluorescence image was first used to highlight the cells in the image, overlapping and too close together cells were removed. Then the cells regions were measured for pixel brightness. The background brightness of the image was also measured and then subtracted from the cell brightness readings from that image. The mean brightness of each cell measure in each image from a given biological replicate treatment was then averaged to give the mean cell brightness within that replicate treatment.

2.1.8 Testing if Fluorescent Strain colony heterogeneity breeds true by visual selection

When observed on agar plates the reporter strains displayed differences from colony to colony in expression of the fluorescent protein, or "brightness." Each of the reporter strains was streaked on a plate of LB agar + Kan for individual colonies and left to grow at 37^{o}C for 18 hours. For each strain they were observed under ultraviolet light. The two brightest and two dimmest colonies for each strain were picked off and streaked again on new LB + Kan plates for individual colonies. These plates were Selection 1, a set of two "bright" plates and two "dim" plates for each of the reporter strains. These plates from Selection 1 were grown overnight at 37^{o}C. Each "bright" plate from Selection 1 was examined for the brightest colony present, which was picked off. Each "dim" plate from Selection 1 was examined for the dimmest colony present, which was picked off. The picked off colonies from Selection 1 were restreaked for individual colonies on LB + Kan plates, thus making up Selection 2, a new set of two "bright" plates and two "dim" plates for each of the reporter strains. These plates were then incubated at 37^{o}C for 18 hours. From the "bright" plates from Selection 2, the brightest colonies were picked off. From the "dim" plates, the dimmest colonies were

picked off. The picked off colonies were each grown up overnight in LB + Kan at in a water bath shaker at 37°C with agitation. These cultures were then each stored in 15% glycerol at -80°C.

These selection regimes resulted in the strains shown in Table 2.3

Table 2. 2: Strains resulting from selection for bright and dim colonies.

Origin Strain	Bright Selected Strains	Dim Selected Strains
BWtolC	B1-B1 B2-B1	D1-D1 D2-D1
BWmarR	B1-B1 B2-B1	N/A
BWsoxS	B1-B1 B2-B1	D1-D1 D2-D1

2.1.9 Determination of the effects of 2,4-D treatment on isolates from different selection regimes

The selected isolates (Bright/Dim Strains) of the reporter strains from section 2.1.8 all could potentially to act as bioreporters of increasing antibiotic resistance when exposed to biocides, but it's possible that not all of them functioned equally well at this task. Prior to section 2.1.9.1 growth rates of BWtolC strains were observed with and without 2,4-D exposure (appendix D).

2.1.9.1 Exposure of Bright/Dim strains to 2,4-D

The Bright/Dim strains were treated as in 2.1.7.2 with the Bright/Dim strains in place of *BWtolC* and 2,4-D (1.95 g/L) in the place of copper ammonium acetate.

Paraformaldehyde fixation is then performed on cells as in 2.1.7.3. Cells are mounted gel slides as in 2.1.6.3.

2.1.9.2 Fluorescence microscopy imaging on Bright/Dim Strains

This analysis was performed as in 2.1.6.4 aside from an alteration in the microscope by the introduction of a new phase contrast objective. Phase contrast images were used in place of the DIC images.

2.1.9.3 Image Processing and Analysis for BWtolC with copper ammonium acetate

This analysis was performed as in 2.1.6.5, but the phase contract images were used to highlight cell regions within images instead of fluorescence images.

2.1.10 Preliminary investigation of the effects of NsARC on BWtolC B2-B1 fluorescence

The effects on NsARC on the fluorescence level of the BWtolC reporter were examined with one replicate due to limited supply of NsARC pieces for testing. The strain was grown to saturation (approximately $1x10^9$ cfu/ml) in LB broth and Kanamycin in a shaker bath at 37°C. The liquid culture was diluted 10-fold in phosphate buffer saline (PBS). 50 µl of dilute culture (approximately 5 million cfu) was placed on NsARC test piece and another 50 µl on glass control. Both test surfaces were in experimental set up as shown in Figure 2.2, with ambient light levels.

They were illuminated for 2 hours in ambient light, same levels as ambient light in section 2.1.5. The bacterial cultures were then each rinsed off in 2 ml of PBS and immediately subjected to paraformaldehyde fixation as in section 2.1.7.2. Cells were then mounted as in section 2.1.7.3. Cell fluorescence was analysed via fluorescence microscopy as in section 2.1.7.4. Fluorescence microscopy images were analysed as in section 2.1.7.5.

2.2 Statistical Analysis

RStudio was used for all ANOVA statistical analyses (RStudio Team, 2015). T-tests were performed and graphs were generated using Graphpad Prism v7.04 (Motulsky, 1999).

2.2.1 Testing of antibacterial properties in UV, ambient and high intensity light

For each of the sections 2.1.4 and 2.1.5 and 2.1.6 the colony counts for each spot on a plate were multiplied by the total dilution factor for that spot, these values were then subjected to the EOP formula in Figure 2.3. The EOP values for each plate were averaged to get an average EOP for each replicate. The resulting EOP averages were analysed in Multifactor ANOVAs using duration of exposure (Time), Light Levels (Light and Dark), and Surface Type (NsARC or Glass) as categories.

2.2.2 Determining the response of fluorescent reporter strains to copper ammonium acetate

For both *BWtolC* and *BWsoxS* separately the cell fluorescence averages from each replicate were analysed in two-tailed T-tests comparing the fluorescence values in just LB broth with LB + copper ammonium acetate.

2.2.3 Determining the response of fluorescent reporter strains to 2,4-D

To test for statistical differences for all strains between exposed and unexposed treatments all strains shown in Table 2.3 had the cell fluorescence averages from each replicate analysed in two-tailed T-tests comparing the fluorescence values in LB broth with LB + 2,4-D. The reporter strains were then grouped together by the strain they were selected from, *BWtolC, BWmarR* or *BWsoxS.* Within each strain they were analysed by two factor ANOVA with Selection Regime as one category and presence/absence of 2,4-D as the other category.

3.0 Investigating the antibacterial properties of NsARC

3.1 Introduction

With TiO_2 coatings the antibacterial mechanism usually involves photocatalytic activity (Muranyi, Schraml, & Wunderlich, 2010). This is achieved using energy from light to pass on electrons and produce reactive oxygen species (ROS), usually via water splitting and producing H_2O_2. ROS then react with the cell membranes and walls, causing breakdown and lysing of the cells (Foster et al., 2011). For most formulations energy in the UV wavelengths is necessary to generate ROS (Pelaez et al., 2012), but the novel formulation being used here was tested for response to visible light too. In this chapter I tested the hypotheses that the NsARC material has greater antibacterial properties than inert surfaces in both UVA and visible light, or NsARC in the dark.

The aims of the work described in this chapter were to:

1- assess whether NsARC's previously tested redox activity was sufficient to cause antibacterial activity in UV light.

2- Investigate whether white light could substitute for UV light when activating NsARC antibacterial activity.

3- Investigate whether the antibacterial activity was from photocatalysis.

To achieve the first aim, I used a UV lightbox and exposed *E. coli* to UV light and darkness. The differences between treatments were used to investigate the antibacterial properties in UV light.

To identify whether NsARC has antibacterial properties in visible light, the experiment above was altered by replacing the UV lightbox treatment with ambient overhead lighting in the lab. The experiment was also repeated with a high intensity visible-light lightbox in place

of the UV lightbox treatment. The comparison of EOPs of the different light exposures on both NsARC and glass can be used to investigate whether light spectrum or intensity is an important factor for NsARC antibacterial effects.

To investigate whether any antibacterial activity could be wholly attributed to photocatalysis the data from the previous 3 experiments can be analysed to look at just NsARC and glass in the dark. This can be used to establish if there is a level of antibacterial effect that could be attributed to an antibacterial quality of NsARC other than photocatalysis.

NsARC surfaces were initially planned to be reusable in these experiments, but preliminary testing showed noticeable decreases in activity in repeated uses. To test the reusability of NsARC under these methods, the testing in ambient light was repeated 3 times to the point of no measurable activity observed. Although this was only a preliminary experiment without replication for statistical analysis it did inform my one-use policy for NsARC test pieces throughout this thesis. It also severely limited the experiments that could be performed because all testing was considered destruction of the surface and the supply was very limited.

3.2 Results

3.2.1 Determining the effects of NsARC on *E. coli BW25113* in different light levels and wavelengths

E. coli ATCC8739 was exposed simultaneously to NsARC or glass and either UV light or no light (dark control). The bacteria were then washed off either immediately (time 0) or after 4 hours, diluted and plated to count colony forming units. Analysis of the EOPs from this was used to investigate first whether NsARC had any antibacterial properties.

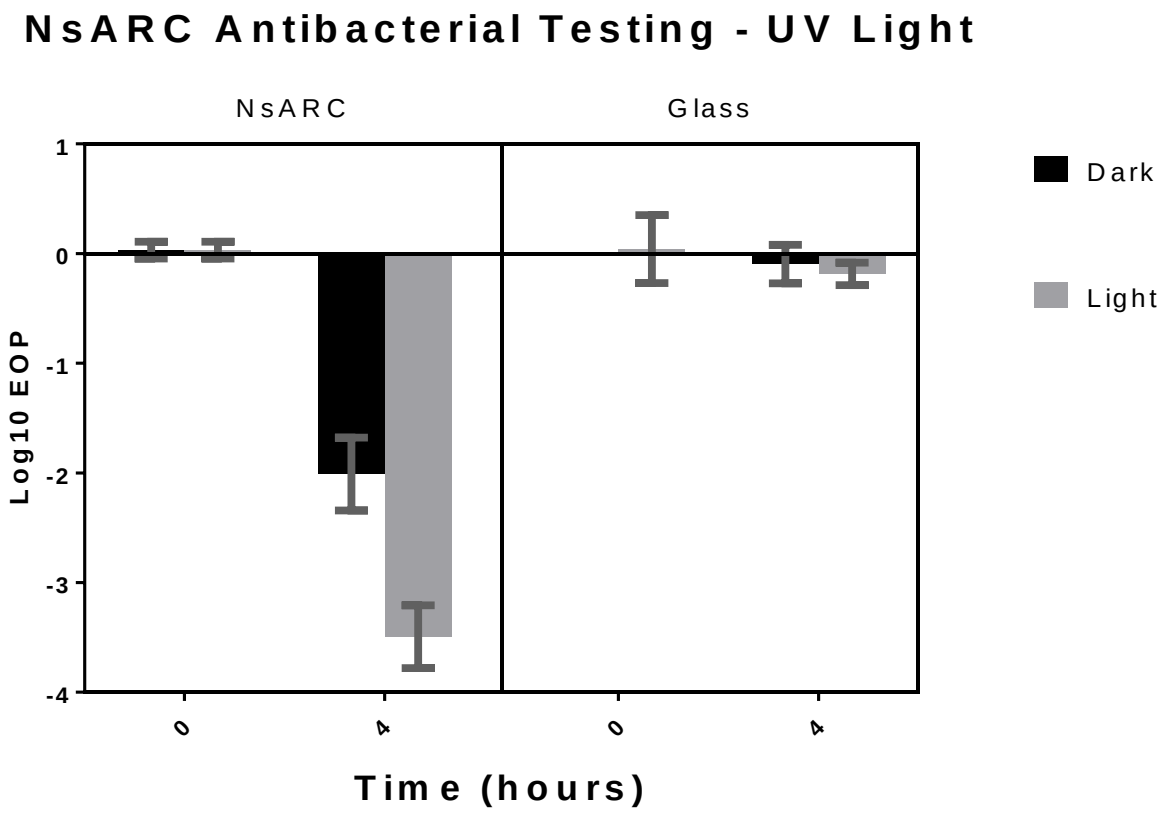


Figure 3. 1: Survival of *E. coli* strain ATCC8739 in UV light or darkness, on NsARC or Glass, for 0 or 4 hours. Survival is reported as Log10(EOP). Error bars are standard deviation (SD).

Table 3. 1: Results of three-way ANOVA analysis. Factors are; Time exposed (0 or 4 hours); Surface exposed (NsARC or glass); Light exposed (365 nm UV light or dark). Significance threshold is P-value < 0.05.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
TIME	34.87	<0.0001
SURFACE	27.23	<0.0001
LIGHT	2.451	0.0008
TIME X SURFACE	26.83	<0.0001
TIME X LIGHT	2.451	0.0008
SURFACE X LIGHT	1.93	0.0021
TIME X SURFACE X LIGHT	1.93	0.0021

Time as a factor on its own was found to have a statistically significant effect on EOP (Table 3.1, row 1), suggesting there was a source of variation from exposure time. This variation could be caused by incomplete recovery of bacteria in wash-off step or limited replication on the glass surface. Surface type on its own was found to have a statistically

significant effect (Table 3.1, row 2). This suggested that surface type was a major source of variation, regardless of time or light. UV light had a statistically significant impact on EOP both on its own and any interaction term Light Levels were involved in (Table 3.1, rows 3, 5, 6) this suggested that UV light is an important factor to changes in EOP. The interaction of time and surface type was statistically significant (Table 3.1, row 4), this can easily be seen in Figure 3.1. NsARC at 4 hours in dark EOP dropped by approximately 100-fold on NsARC. After 4 hours in UV light EOP dropped by over 1000-fold. Both NsARC in UV Light and Dark treatments at 4 hours show a significant antimicrobial effect compared with glass controls that had showed no more than a 10-fold change in EOP.

Activity in ambient wavelengths would be an advantage for material used on commonly touched surfaces in hospitals. To investigate if there was any observable difference produced by illumination from a different light spectrum, the experiments above were repeated using ambient indoor lighting (fluorescent bulbs 400-800 nm with the peak around 650 nm and approximately 750 lux).

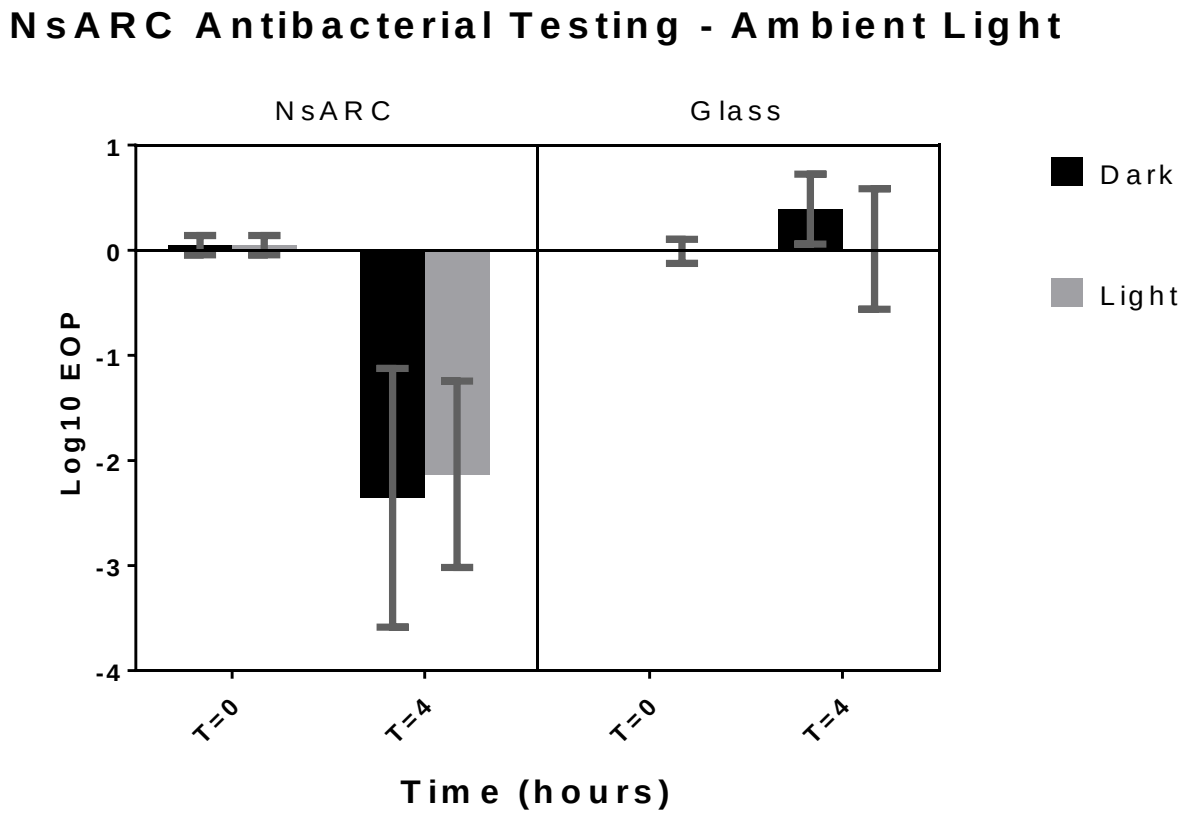


Figure 3. 2: Survival of E. coli strain ATCC8739 in ambient light or darkness, on NsARC or Glass, for 0 or 4 hours. Survival is reported as Log10(EOP). Error bars are standard deviation (SD).

Table 3. 2: Results of three-way ANOVA analysis. Factors are; Time exposed (0 or 4 hours); Surface exposed (NsARC or glass); Light exposed (750 lux visible light or dark). Significance threshold is P-value < 0.05.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
TIME	21.41	0.0005
SURFACE	28.2	0.0001
LIGHT LEVELS	0.02999	0.8736
TIME X SURFACE	31.07	<0.0001
TIME X LIGHT LEVELS	0.02999	0.8736
SURFACE X LIGHT LEVELS	0.4472	0.5414
TIME X SURFACE X LIGHT LEVELS	0.4472	0.5414

Time as a factor on its own was found to have a statistically significant effect on EOP (Table 3.2, row 1), suggesting there was a source of variation from exposure time. Once again this is most likely just due to incomplete wash-off of bacteria or limited replication on glass. Surface type on its own was found to have a statistically significant effect (Table 3.2,

row 2). This suggested that surface type was a major source of variation, regardless of time or light. Ambient light was found to be a statistically nonsignificant factor on EOP, both singularly and in combination with Time or Surface Light (Table 3.2, row 3, 5 and 6). This suggests that ambient light levels may not be an important factor to changes in EOP. Time and Surface Type as a combined factor was also shown to be statistically significant (Table 3.2, row 4), this effect was less pronounced than in the UV light experiment, as shown in Figure 3.2. The variation among replicates was much higher in this experiment than in the equivalent experiments where the light source was in the UV range, but killing was statistically significant. At 4 hours replicates of NsARC in dark and NsARC in Ambient Light both dropped EOP by approximately 30-fold to 40-fold. In comparison glass controls at 4 hours in light and dark showed an increase in EOP. This suggests that NsARC had an antibacterial effect, but light had no additive effect.

It is possible that light intensity is more important to antibacterial activity than wavelength for NsARC. To investigate if there was any observable difference produced by illumination from a different light intensity, I changed the intensity of the light in the visible spectrum using a high intensity lightbox (fluorescent bulbs 400-800 nm with peaks around 450 nm and 600 nm and 2100 lux).

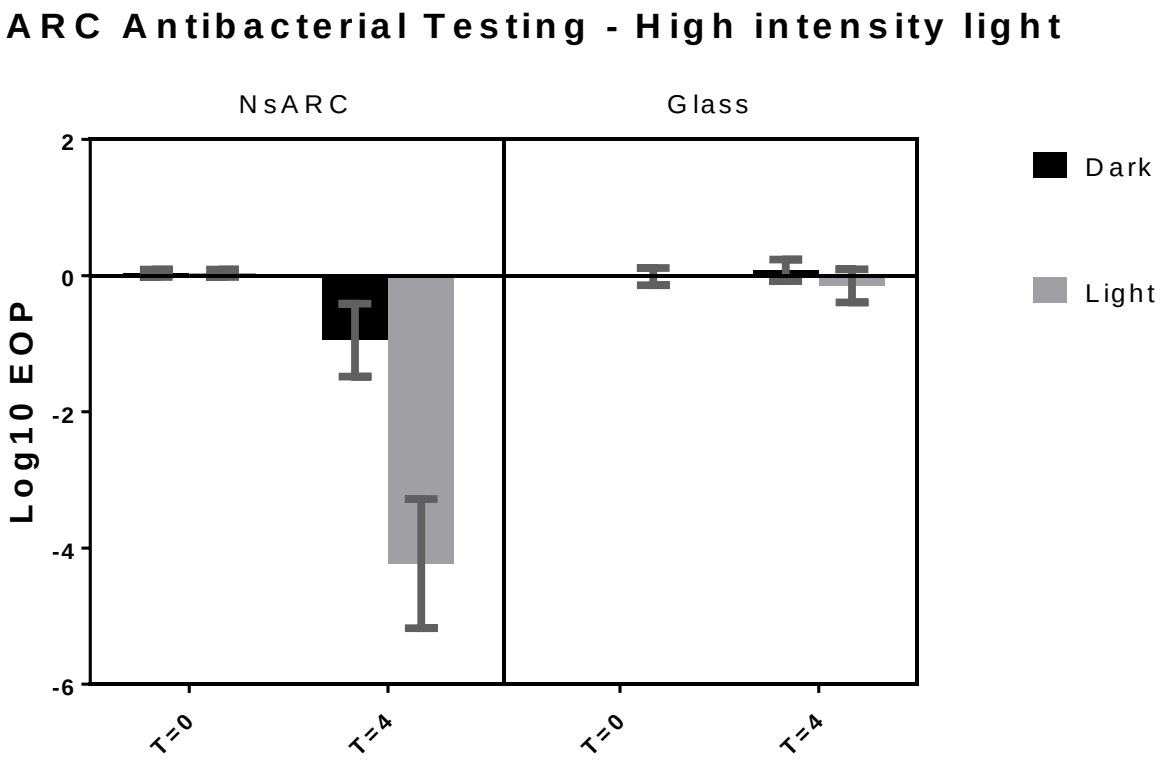


Figure 3. 3: Survival of E. coli strain ATCC8739 in high intensity visible light or darkness, on NsARC or Glass, for 0 or 4 hours. Survival is reported as Log_{10}(EOP). Error bars are standard deviation (SD).

Table 3. 3: Results of three-way ANOVA analysis. Factors are; Time exposed (0 or 4 hours); Surface exposed (NsARC or glass); Light exposed (2100 lux visible light or dark). Significance threshold is P-value < 0.05.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
TIME	21.45	<0.0001
SURFACE	19.22	<0.0001
LIGHT	9.464	<0.0001
TIME X SURFACE	20.77	<0.0001
TIME X LIGHT	9.464	<0.0001
SURFACE X LIGHT	7.147	0.0003
TIME X SURFACE X LIGHT	7.147	0.0003

2 out of 3 replicates of NsARC in light at 4 hours showed no growth. Plating error was a possibility, but the detection limit was decreased by 10-fold for the final replicate of the experiment. The final replicate produced countable colonies just above the detection limit.

This suggests that the first two replicates had an antibacterial effect that dropped cfu/ml below the detection limit of -3 Log_{10}EOP. For the two replicates that produced no countable cfus the detection limit was substituted for the plate counts for the purposes of statistical analysis.

Time as a factor on its own was found to have a statistically significant effect on EOP (Table 3.3, row 1), suggesting there was a source of variation from exposure time. Surface type on its own was found to have a statistically significant effect (Table 3.3, row 2). This suggested that surface type was a major source of variation, regardless of time or light. Light had a statistically significant factor on EOP in this experiment both singularly and in combination with Time and Surface terms (Table 3.3, row 3, 5 and 6), this suggested that light levels may be an important factor to changes in EOP. Interaction of Time and Surface was also shown to be statistically significant (Table 3.3, row 4), this effect is still pronounced, as shown in Figure 3.3. The variation among replicates is still higher in this experiment compared to equivalent experiments in UV Light, but this may be due to 2 replicates being recorded at detection limit. The antimicrobial activity and the impact of light remains statistically significant. At 4 hours NsARC in dark treatment dropped EOP by approximately 10-fold. At 4 hours NsARC in high intensity visible Light dropped EOP by approximately 10000-fold compared to 0-hour controls. In comparison glass controls at 4 hours in light and dark showed minimal changes. This suggests that NsARC has an antibacterial effect and that light increases this effect.

3.2.2 Determining the effects of NsARC on *E. coli* ATCC8739 in darkness

Intensity was found to be the critical factor for NsARC antibacterial activity in the visible spectrum. This raised the hypothesis that NsARC has a significant effect on EOP in

darkness. Previous research on low light intensity photocatalytic activation appears to only go as low as ~10 µW/cm^2 ("a reasonably well lit room") (Fujishima, Rao, & Tryk, 2000; Konstantinou & Albanis, 2004), but the authors state that activity was still present at these low light levels and potentially lower. To establish what this low-light level of activity is, all sample data from Dark treatments from the 3 experiments was pooled and analysed in a two-way ANOVA analysis with 9 replicates per treatment.

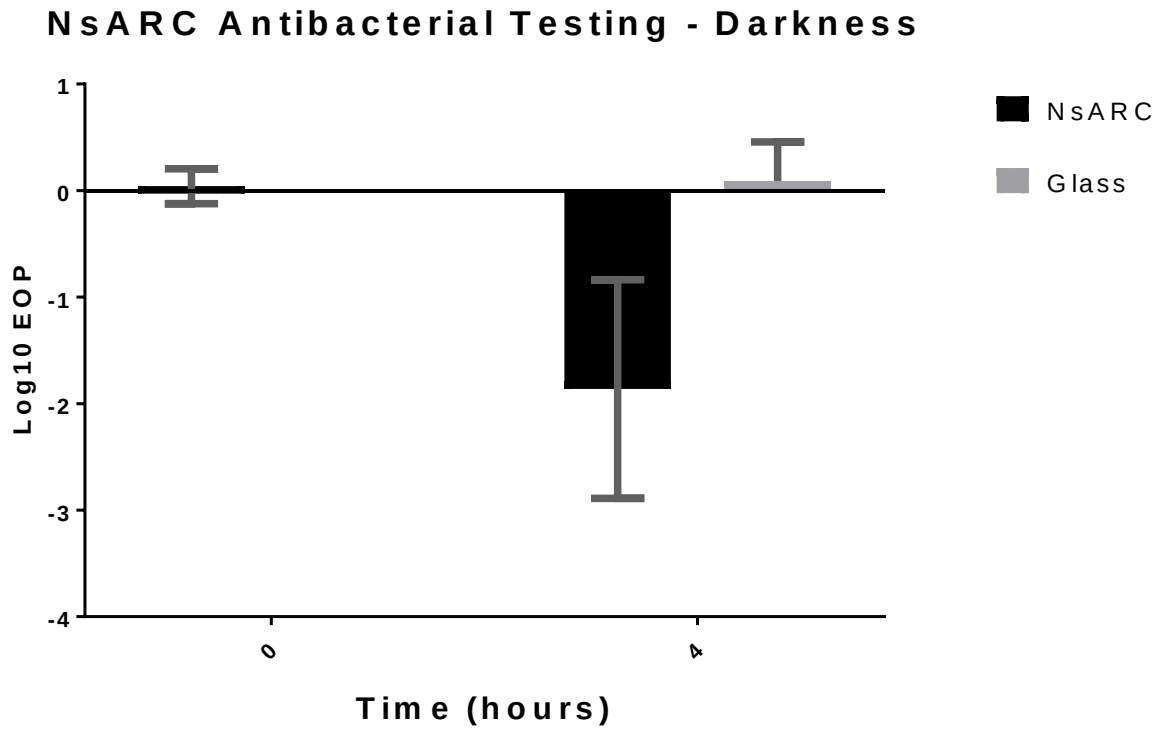


Figure 3. 4: Survival of E. coli strain ATCC8739 in darkness, on NsARC or Glass, for 0 or 4 hours. Survival is reported as Log_{10}(EOP). Error bars are standard deviation (SD).

Table 3. 4: Results of two-way paired ANOVA analysis. Factors are; Time exposed (0 or 4 hours); Surface exposed (NsARC or glass) Significance threshold is P-value < 0.05. Observations within the same replicate were treated as paired values.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
INTERACTION	26.72	<0.0001
TIME	21.03	0.0001
SURFACE	23.27	0.0002
SUBJECTS (MATCHING)	13.65	0.5901

The ANOVA analysis shows that the interaction value for time and surface is significant and accounts for the highest proportion of variation (table 3.4). The magnitude change in all treatments on glass at 4 hours is down to levels that are nonsignificant. NsARC exposure in the dark for 4 hours on the other hand clearly dropped EOP by a mean of approximately 100-fold.

3.2.3 Does NsARC lose activity over time?

I noticed that samples of NsARC sharply decrease in antibacterial activity with repeated use. It was this observation that resulted in the protocol of only using fresh samples for experimentation, even though this resulted in supply delays. I thus hypothesised that NsARC test piece use results in decreased activity in following uses. This was initially tested with the sample set that was used for NsARC antibacterial activity testing in ambient light. The sample pieces were washed in 70% ethanol following use and allowed to air dry. Following this they were stored in a sealed container and kept in the dark for 2 days to exhaust any ongoing photocatalytic activity. The pieces were then treated as a new set would be and tested as previously described in section 2.1.4. The only measurements graphed are 4-hour light exposures for both NsARC and glass.

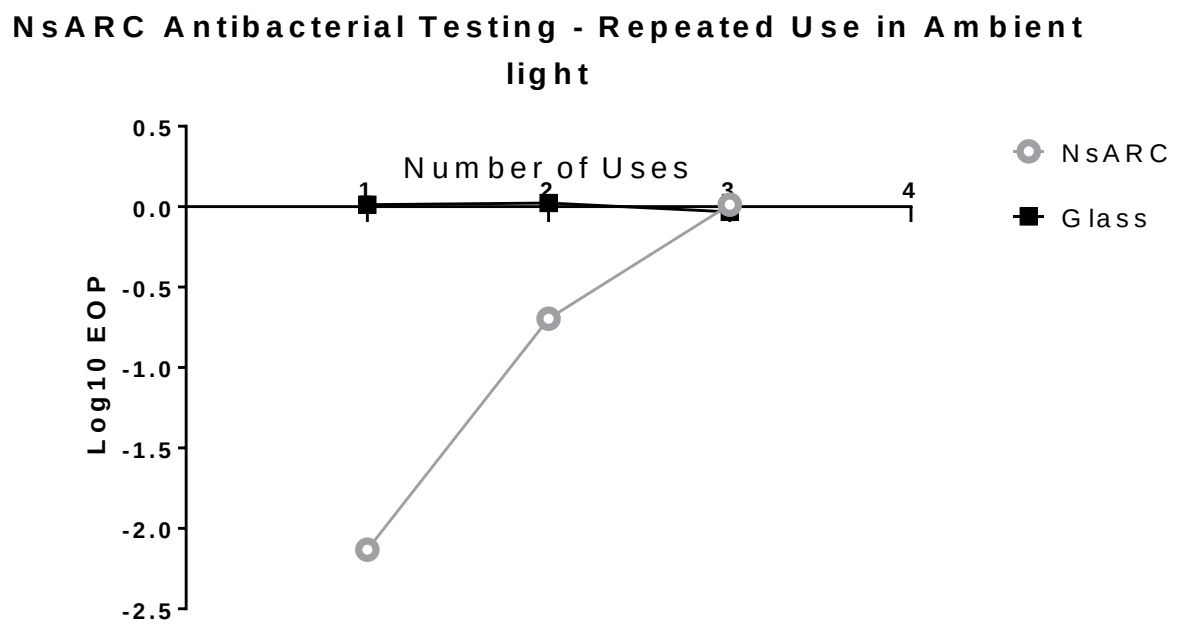


Figure 3. 5: Survival of E. coli strain ATCC8739 on NsARC after 4 hours in ambient visible light (750 lux) with repeated uses of NsARC surface set. Survival is reported as Log10(EOP).

Antibacterial activity decreased as a function of repeated use of NsARC test pieces. As shown in Figure 3.5, the Log_{10}EOP after 3 cycles of exposure to NsARC approaches 0. This experiment of the repeated use of NsARC was only performed once due to limitations on NsARC supply. However, the trend seems clear and suggests that after 2-3 uses there is no measurable difference between the antibacterial activity of NsARC and Glass.

3.3 Discussion

Many pathogens survive for long periods of time on surfaces and spread should those surfaces be touched. Many Gram-positive bacteria, including some *Enterococcus* and *Staphylococcus,* are capable of surviving on dry surfaces for months (Kramer et al., 2006). This is why hygiene in healthcare remains a large expense and has consistently increased in costs as cleaning practices become more stringent (Ministry of Health NZ, 2012). Even with stringent cleaning practices, rates of and costs from nosocomial infections are high, estimated in the United States of America at $16.6 billion in 2010 (Hassan, Tuckman, Patrick, Kountz, & Kohn, 2010). One method that has been used to limit the survival of pathogens on

surfaces is making the surfaces from antibacterial materials. Copper for example has a long history of being used in metallic alloy forms due to antimicrobial properties (Dollwet & Sorenson, 1985; Kuhn, 1983; Mehtar, Wiid, & Todorov, 2008; Vincent, Hartemann, & Engels-Deutsch, 2016).

Research has gone into photocatalytic surfaces, especially TiO_2, due to their high oxidative activity which can be used for antimicrobial activity and relatively low costs (Campoccia, Montanaro, & Arciola, 2013; Foster et al., 2011; Zhao, Milanova, Warmoeskerken, & Dutschk, 2012). But a problem that has been faced with the use of TiO_2 is that its activity is caused by UV light, this limits its usefulness in indoor settings due to a lack of UV light indoors (Foster et al., 2011). Recent research has gone into how to change the light wavelength by which photocatalytic surfaces are excited. Change in the excitation wavelength has primarily been achieved by combining TiO_2 with carbon, nitrogen, or sulphur, or other metals such as Sn, Pd and Cu (Fujishima & Zhang, 2006). Some of these approaches have been shown to work and provide photocatalytic activity in visible light and even shown antimicrobial activity in visible light (Ahmad et al., 2015; Caballero, Whitehead, Allen, & Verran, 2009; Q. Li et al., 2007; Wong et al., 2006). A surface coating that could demonstrates visible light antimicrobial activity and is inexpensive to produce would be beneficial for lowering risks of nosocomial infections. The effects of such an antimicrobial surface have been shown in a clinical setting by testing with copper alloys (Karpanen et al., 2012; Schmidt et al., 2012).

3.3.1 NsARC has antibacterial properties in UV light

TiO_2 based materials have been previously shown to be photocatalytically active in UV light (<400 nm) (Foster et al., 2011) and some early testing performed on NsARC with

the degrading of oxidation sensitive dye was performed under UV light. I performed initial antibacterial testing of NsARC using UV light to investigate whether NsARC had antibacterial properties at all.

EOP values were used to determine the antimicrobial activity of NsARC after 0 or 4 hours in the dark or with additional exposure to UV light. The comparator was always glass. After 4 hours in UV light on NsARC the average EOP was approximately 1000-fold lower than that of glass. After 4 hours in the dark the average EOP from NsARC dropped by approximately 100-fold. Glass after 4 hours in darkness and UV light showed minimal changes in EOP. The ANOVA analysis shows that Time and Surface are the most significant factors, but all interactions between factors Time, Surface and Light were shown to be significant. This result suggests that NsARC does have an antimicrobial effect. The antimicrobial effect of NsARC is comparable to other TiO_2 coatings. For example, Xu et al. (2006) found that their formulation of N-TiO_2 inactivated 75% of *Bacillus subtilis* in 70-80 minutes. If that rate of death was sustained for 4 hours then the number of live bacteria would have dropped almost 1000-fold, comparable with the activity of NsARC I observed under comparable conditions. Muranyi et al. (2010) found that after 4 hours of UV illumination the bacterial count of *Kocuria rhizophilia* dropped by 3.3 orders of magnitude, almost exactly the same as the findings in this thesis with *E. coli*.

The antibacterial effects of NsARC appear comparable with previous work on TiO_2 films in UV light, but the activity in darkness suggests that there is another mechanism at work that may be contributing to photocatalytic activity or there may be photocatalytic activity present at very low light levels too. Light and Light-Time interaction were found to be significant factors. This suggests that the UV light had a statistically significant killing effect regardless of surface type. This is understandable as UVA light (wavelengths from 320

nm to 400 nm) has been shown to have limited biocidal properties (Bintsis Thomas, Litopoulou-Tzanetaki Evanthia, & Robinson Richard K, 2000). NsARC was shown to have a statistically significant antibacterial effect in the dark. The cause of this effect required further investigation.

3.3.2 NsARC antibacterial properties in white-light

Previous formulations of TiO_2 have shown antimicrobial activity in the visible light spectrum (Caballero et al., 2009; Q. Li et al., 2007; Pal, Pehkonen, Yu, & Ray, 2007; Pelaez et al., 2012). The importance of this activity should not be understated and would allow photocatalytic surface coatings to move from a specialist tool, to a generally used technology aiding prevention of pathogen touch-transmission. Previous testing of NsARC using oxidation degradable dye, indicated photocatalytic activity in visible light as well as UV light. Given that the eventual goal with NsARC use is use in visible light, it required testing under visible light conditions. Visible light testing was done at light intensities found indoors with commonplace fluorescent overhead light (approximately 700 lux) and using a fluorescent lightbox (a higher intensity light 2100 lux, approximately the same as that in a filming studio). Two light levels were tested to investigate if light levels may be a factor of antibacterial activity.

EOP values were used to determine the antimicrobial activity of NsARC in 4 hours darkness and ambient light compared to glass under the same conditions. After 4 hours in ambient light the average EOP from NsARC dropped by approximately 30-fold. After 4 hours in darkness the average EOP of NsARC dropped by 40-fold. There was no statistically significant effect from ambient light. The EOPs of Glass after 4 hours in darkness and ambient light displayed minimal changes, with the EOP of glass in darkness even increasing

slightly. The ANOVA analysis shows that Time and Surface are the most significant factors, but with the only significant interaction term being Time and Surface. Light, and any interaction terms containing light showed no significance. This result suggests that NsARC does have an antimicrobial effect, but it is not dependent on light at 700 lux.

The EOP values were used to determine the antimicrobial activity of NsARC in 4 hours darkness and 2100 lux light compared to glass under the same conditions. After 4 hours in 2100 lux light the average EOP from NsARC dropped by approximately 10000-fold, while after 4 hours in darkness the average EOP from NsARC dropped by approximately 10-fold. The EOPs of Glass after 4 hours in darkness and 2100 lux light displayed minimal changes. The ANOVA analysis shows that Time and Time X Surface are the most significant factors, but all interactions between factors, Time, Surface and Light, are shown to be significant as well. This suggests that NsARC does have an antimicrobial effect in visible light at 2100 lux and it is at least partially dependent on light at these levels. Comparing the results from the two experiments using visible light suggests that photocatalytic activity is occurring in the visible light spectrum but is not significant at indoor ambient intensities (700 lux).

This result is comparable with past work on visible light antimicrobial activity of photocatalytic surfaces. Pal et al. (2007) showed 40-50% inactivation of *E. coli* following 2 hours of exposure to fluorescent lighting on a TiO_2 surface, significantly less activity than was shown with NsARC in 2100 lux lighting. This difference could be due to different light intensities, but it seems likely that NsARC still has greater antimicrobial activity due to whatever is causing the antimicrobial activity in darkness. Caballero et al., (2009) showed total inactivation of *E. coli* after 2 hours with varying concentrations of TiO_2. This activity seems to outstrip NsARC considerably, it may be due to the differing concentrations of TiO_2.

The higher activity may also be due to the TiO_2 surface in Caballero et al., (2009) being generated by TiO_2 being dried on a membrane filter rather than annealed to the surface in an anatase-rutile structure as with NsARC. In the discussion in the paper, dried powder substrate is suggested as being able come loose and enter cells to enhance killing effects (Caballero et al., 2009) and this may enhance antimicrobial activity far more than annealed surfaces. The effect of the substrate coming loose would also mean that the material is not stable or long lived though.

Given the higher antimicrobial activity of NsARC in higher light intensities this suggests at least some of the antibacterial activity is from photocatalytic activity. Caballero et al. (2009) suggest different light levels used in the studies as an explanation for different antimicrobial activities at similar TiO_2 concentrations.

3.3.3 NsARC has antibacterial qualities in the absence of light

NsARC formulation is based on TiO_2 and this material is known primarily for its oxidative activity caused by photocatalysis. This does little to explain the antimicrobial activity that was consistently observed when bacteria were exposed to NsARC in darkness. A two-way ANOVA analysis was performed on all replicates exposed to NsARC in darkness compared to those of glass under the same conditions. This shows that there is a significant antimicrobial effect from NsARC in darkness.

Previous research has shown little to no oxidative activity with TiO_2 in darkness, using the lack of degradation of toluene by TiO_2 in darkness as an assay method (Sekiguchi, Sanada, & Sakamoto, 2003). With live bacteria a formulation of TiO_2 in anatase phase in the dark has been shown to have antibacterial activity, this activity was also enhanced by light exposure (Akhavan, 2009) similar to the findings in this thesis. Conversely no antimicrobial

effect was seen in darkness while using pure TiO_2 with anatase phase in other research (Armelao et al., 2007). The formulation and structure of TiO_2 can have a great effect on its oxidative and antimicrobial activity, TiO_2 can have 3 main structures in a surface or a mixture of them, anatase, rutile or brookite (J. Zhang, Zhou, Liu, & Yu, 2014). But given that Akhavan, (2009) and Armelao et al., (2007) both used formulations of TiO_2 with primarily anatase structure and obtained different results in the dark suggests that there is a reason other than the phase for the differences. Different annealing temperatures and morphology of TiO_2 can have a significant effect on the activity of the resultant surface, if the two methods were performed under different conditions the different TiO_2 morphology and activity of the surfaces could produce different results (Mathews, Morales, Cortés-Jacome, & Toledo Antonio, 2009). Different TiO_2 concentrations, annealing temperatures and morphology make-up make direct comparison between NsARC and other photocatalytic films difficult. But previous research on photocatalytic film does establish that antimicrobial activity in the dark is not unprecedented and some even suggest that it can be attributed to direct contact with nanocomposite structures having an antimicrobial effect (Adams, Lyon, & Alvarez, 2006; Kubacka, Muñoz-Batista, Ferrer, & Fernández-García, 2013).

The light intensities NsARC was stored in or used at in dark treatments (approximately 1 lux) in a dim drawer are unlikely to cause photocatalytic activity. Photocatalytic activity in the dark is unlikely due to an effect known as recombination, this is when the electrons and holes generated by the surface recombine and the energy released is simply dissipated as heat (Liu Jincheng et al., 2010). This phenomenon is overcome at higher levels of light exposure but at lower levels, much of the energy hitting a photocatalytic surface is simply wasted (Choi, Termin, & Hoffmann, 1994). Due to these effects it is unlikely to be photocatalytic activity at low light levels causing antimicrobial

activity. The possibility of photocatalytic activity in low light intensities can be tested by testing of the material in darkness for oxidative activity using oxidation degraded dyes such as methylene blue.

NsARC is a TiO_2 surface doped with carbon; TiO_2 powders interacting with C-H bonds have been shown to generate carbon-centred oxidative activity in the dark (Fenoglio Ivana, Greco Giovanna, Livraghi Stefano, & Fubini Bice, 2009, p.). Carbon centred oxidative activity, combined with direct toxicity of nanocomposite material might explain the killing in the dark. The possibility of carbon redox activity could be tested. If the material still appears to be undergoing production of reactive oxidation species in the dark, then this would suggest the presence of carbon-centred oxidative activity. The removal of carbon from the film and testing antimicrobial activity in darkness would be possible. If antimicrobial activity is still present it would suggest a toxic effect from contact with nanostructured materials because in all instances the surface morphology remains unchanged.

3.3.4 NsARC surfaces decrease in activity with repeated use

From the repeated use of one set of NsARC pieces (section 3.2.3) I observed a trend of decreased antimicrobial activity over time. This effect was observable in all 40 NsARC test pieces, but the full experiment was only performed once. The fact that test pieces were not reusable had a substantial effect on this research project by imposing limitations on the rate of experimentation due to limited supply of test pieces. The effect appeared to be caused by the high salt content of the suspension which *E. coli* were in when they were exposed to NsARC. The NsARC coatings were on stainless steel pieces. Stainless steel under normal circumstances is resistant to corrosion and will take a substantial period of time to show any effect (Scotto, Cintio, & Marcenaro, 1985). The decreased activity of NsARC was theorised to be due to oxidative activity speeding up corrosion of stainless steel under the NsARC surface

and causing NsARC to flake off around cracks and holes in the surface first as these areas would be the first to interact with the salt solution. This has been seen with other TiO_2 uses (Głuzek, Masalski, Furman, & Nitsch, 1997). The flaking off effect was visible in many pieces following their use. A solution to this problem has been suggested by putting the NsARC coating on glass or other inert materials. But it is worth noting that the substrate upon which NsARC is coated has a significant effect on the structure and properties of the surface.

In conclusion, this study demonstrated antibacterial activity from NsARC in UV, visible light, and in the dark (to a lesser degree). This study has shown that at least some of the activity can be attributed to photocatalysis. The activity of NsARC in darkness requires further investigation but seems attributable to one or more of several previously observed phenomena.

4.0 Measuring the adaptive response as an indicator of adverse NsARC effects

4.1 Introduction

Worryingly, it may not be just the overuse of antibiotics that is causing changes in antibiotic resistance; the overuse of many commercial biocides can cause these changes too (Fernández, Breidenstein, & Hancock, 2011; Kurenbach et al., 2015). Previous work by my research colleagues added commercial formulations of several herbicides to the list of commercial chemicals that at sub-lethal concentrations could induce changes in response to antibiotics (Kurenbach et al., 2015). Furthermore, these changes were shown to be caused by purified active ingredients and surfactants (Kurenbach et al 2017).

Resistance is categorised as intrinsic or acquired. Intrinsic resistance is due to the genotype of the organism and is uniform in a species (Cox & Wright, 2013). A good example is *Proteus* intrinsic resistance to polymyxins, which is due to their altered lipopolysaccharides (Olaitan et al., 2014). Adaptive resistance is intrinsic too, but environmentally induced. There are many ways the environment can change to induce this, changes such as cues from other organisms can do this (Levin & Rozen, 2006) or sub-lethal concentrations of biocidal chemicals (Fernández et al., 2011; Heinemann, Ankenbauer, & Amábile-Cuevas, 2000; Kurenbach et al., 2017). Acquired resistance is a change in genotype brought about by gene acquisition or mutation (Hoek et al., 2011).

One of the primary aims of this project was to investigate the possibility that NsARC causes an adaptive response. Therefore, I'll now provide relevant background on adaptive resistance. This is caused by changes in gene expression that usually results in a decreased influx and increased efflux of drugs (Heinemann et al., 2000), for example by

downregulation of porin synthesis or upregulation of efflux pump expression. For example a change in efflux regulation has been reported for exposure of *E. coli* to salicylate (Price, Lee, & Gustafson, 2000). Salicylate was shown to upregulate the *marRAB* operon. The *mar* (multiple antibiotic resistance) operon codes a transcriptional regulator for a range of genes involved in stress response, which have the overall effect of decreasing biocide influx and increasing the amount of antibiotic needed to kill the bacterium (Dupont, James, Chevalier, & Pagès, 2007; Fernández & Hancock, 2012). When a bacterium is exposed to a stressor, the repressor, MarR ceases binding with the promoter region of the operon. *marA* is the gene for a signalling protein which is then transcribed. It constitutes the main effector of expression levels of several genes in the network, including porins genes (e.g. *ompX* and *ompF*) and efflux pump components (e.g. *acrAB* and *tolC*). These changes in gene expression have the overall effect of decreasing biocide influx and increasing the amount of antibiotic required to kill the bacterium (Dupont et al., 2007; Fernández & Hancock, 2012).

While adaptive resistance increases the minimum inhibitory concentration of an antibiotic, the increase is often less than what is caused by a mutation or horizontally acquired genes (Russell A.D., 2002). If all that mattered was whether or not a change in resistance was beyond the levels of clinical treatment, then adaptive resistance would not be important. However, smaller changes in antibiotic resistance can make larger changes via mutation or gene acquisition more likely (Fernández et al., 2011). Adaptively resistant phenotypes making acquired resistance more likely can commonly been seen in biofilms, as conjugation and gene transfer are more common in these forms (Driffield, Miller, Bostock, O'Neill, & Chopra, 2008; Hausner & Wuertz, 1999; Molin & Tolker-Nielsen, 2003). The effect has also been well studied in *Pseudomonas aeruginosa* where adaptive resistance by changes of efflux can lead to the bacteria surviving at higher concentrations. Survival of *P.*

aeruginosa while afflicted by an antibiotic at sub-lethal concentrations means selection pressure for a resistant mutation is present, therefore any mutation incurring higher resistance is actively being selected for (Breidenstein, de la Fuente-Núñez, & Hancock, 2011; Fernández & Hancock, 2012).

Small changes in resistance can have a significant effect on treatment outcome for patients with a bacterial infection. This is because treatment of a given species is based on government advice on regional phenotypes and small changes in this can lead to dosage being insufficient. This was seen in treatment of *Salmonella entericia* with decreased ciprofloxacin susceptibility in patients returning from overseas. The insufficient dosage based on assumed resistance levels lead to increased hospital stay compared with patients infected with *S. entericia* with the higher susceptibility levels used to determine the dosing advice (Hassing et al., 2013).

In this chapter I outline the design and use of 3 reporter bacteria with the purpose of detecting changes in the adaptive response when the reporter bacteria are exposed to chemical inducers known to change expression of efflux genes. The genes selected for use in the reporter strains were *tolC*, *marR*, and *soxS* because they can be seen as indicators of an adaptive response.

tolC

In *E. coli*, the AcrAB-TolC protein complex is part of the RND efflux pump family. This pump assembly comprises the outer-membrane channel TolC, the secondary transporter AcrB located in the inner membrane, and the periplasmic AcrA, which lies between membranes and connects between TolC and AcrB. The AcrAB-TolC efflux pump can transport compounds with little chemical similarity, thus conferring resistance to a

broad spectrum of antibiotics and other substances (Baucheron et al., 2004; Du et al., 2014; Motta et al., 2015). It has been found to increase the MIC of many antibiotics including tetracycline, chloramphenicol, fluoroquinolones, rifampicin, and β-lactams (Li & Nikaido, 2004; Nikaido, 1996). This efflux pump complex and many others like it are under tight control by transcriptional regulators. Transcription of the *tolC* gene is activated by the binding of one of several transcription regulators onto a region in the *tolC* promoter known as the 'marbox' (A. Zhang, Rosner, & Martin, 2008). Induction is known to be triggered by the biocidal metal copper as this can induce *tolC* transcription by indirect induction of *arcD* transcription (Nishino, Nikaido, & Yamaguchi, 2007). The induction of *acrD* is due to the use of similar promoters by *tolC* and *cusC,* which codes a copper specific membrane channel protein (Franke, Grass, Rensing, & Nies, 2003). TolC can be part of several efflux pump systems (e.g. AcrAB-TolC, AcrEF-TolC, EmrAB-TolC, MacAB-TolC) (Kobayashi, Tsukagoshi, & Aono, 2001; H. T. Lin et al., 2009; Morona, Manning, & Reeves, 1983; Touzé et al., 2004; Zakharov, Sharma, Zhalnina, Yamashita, & Cramer, 2012), Therefore it makes a good indicator of adaptive resistance because these pumps can react to different triggers. It is because of these responses that it was selected to be part of this study.

marR

The multiple antibiotic resistance (*marRAB*) operon in *E. coli* was discovered during research of tetracycline resistance and is used as a model of intrinsic resistance from antibiotics (Ariza, Cohen, Bachhawat, Levy, & Demple, 1994; Prajapat et al., 2015). The *marRAB* operon is controlled via positive and negative feedback control. The proteins MarA and MarR are DNA-binding transcriptional regulatory proteins (Prajapat et al., 2015). When lacking any stress signals MarR binds to the *marRAB* promoter and negatively regulates gene

expression (Martin & Rosner, 1995). When inducers like salicylate and other phenolic compounds are present, MarR binds to them and is then unable to bind to the *marRAB* promoter (Cohen et al., 1993). Copper has been shown to be directly involved in MarR activation because this protein acts as a copper sensor (Hao et al., 2014). MarA acts as an activator of the operon by binding to the *marRAB* promoter (Alekshun & Levy, 1999). Other target genes of MarA are regulated positively or negatively and control physiology in several ways that tend to increase cell survivability when subjected to stress (Prajapat et al., 2015). The effects on the genes targeted by MarA include upregulation of efflux pumps, including *acrAB* and *tolC*, downregulation of porin *ompF* to decrease membrane permeability. It is because of its association with adaptive resistance that it was selected to be part of this study.

soxS

SoxS is another transcription factor and a homologue of MarA (Storz & Imlayt, 1999). SoxS regulates many proteins that defend *E. coli* from oxidative stress, these include superoxide dismutase (SOD), DNA repair endonuclease IV, glucose 6-phostphate dehydrogenase (Nunoshiba et al., 1992) and, just like MarA, the *acrAB* efflux system (Gu & Imlay, 2011). Over-expression of *soxS* increases antibiotic resistance (Greenberg et al., 1990). Others have hypothesised that SoxR acts as a redox sensor and is induced by superoxide (O_2^-), often generated by chemical attack on the cell (Zheng et al., 1999). This induction would cause SoxR to bind to the *soxS* promoter and induce transcription of this transcription factor which alters expression in genes associated with redox defence (Storz & Imlayt, 1999). More recent research shows evidence to support the theory that SoxR is not activated by superoxide, but by “redox-cycling drugs” (e.g. paraquat or menadione). This was shown by exposing *E. coli* to excess superoxide and it not effectively activating SoxR in a

superoxide dismutase deficient (SOD^-) mutant, while an overexpressing SOD mutant was not able to prevent SoxR activation by "redox-cycling drugs" (Gu & Imlay, 2011). *soxS* has been shown to be induced by several herbicides, leading to an adaptive response to copper exposure, with increases in expression by up to threefold (Yamamoto & Ishihama, 2005). It is because of this association with adaptive resistance that it was selected to be part of this study.

Aims

I am interested in evaluating potential adverse effects of NsARC should it be commercialised. One possible adverse effect is that it selects for bacteria that are resistant to clinical antibiotics, as was shown for chemical agents such as herbicides. In that case, herbicides caused an adaptive response leading to a multidrug resistance phenotype (Kurenbach et al., 2015, 2017). To test whether a ROS generating agent could also cause adaptive resistance to antibiotics, I designed and constructed strains that 'report' when *tolC*, *marR*, or *soxS* expression changes (Figure 4.1).

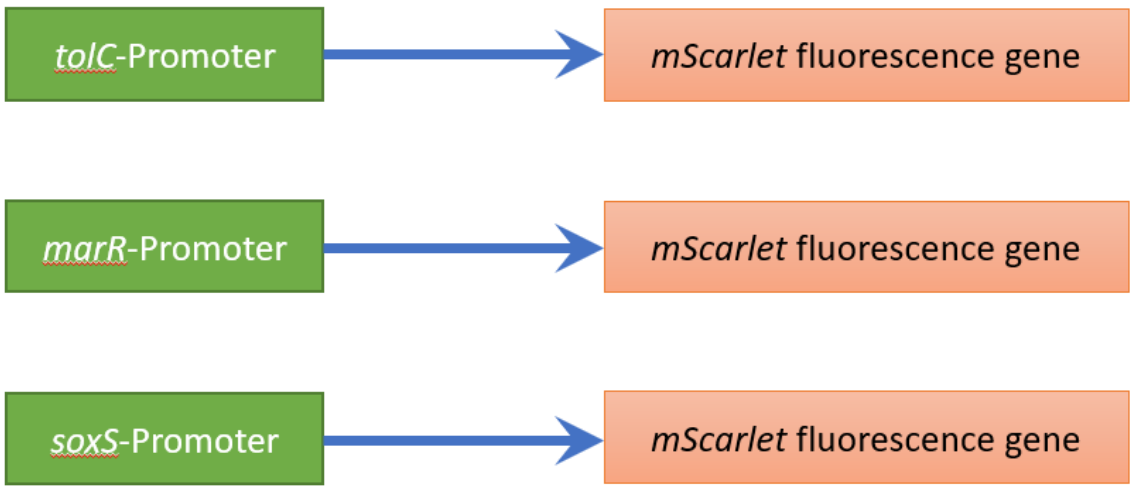


Figure 4. 1: Layout of relevant regions of plasmid gene constructs. Promoter regions from the genes of interest (*tolC*, *marR*, and *soxS*) placed to control expression of the *mScarlet* fluorescent protein.

There is some basis for making such an association. Resistance to ROS has been demonstrated with comparisons of photocatalytic effects on vancomycin-resistant *Enterococcus faecalis* compared to vancomycin-susceptible *E. faecalis* (Tsai Ting-Mi et al., 2010). Resistance to oxidative activity via induction of adaptive response genes, including efflux genes, is well studied (Wang et al., 2010) and it has been suggested as an explanation for different resistances to photocatalytic activity between different bacterial species (Nahim-Granados, Sánchez Pérez, & Polo-Lopez, 2017).

In this chapter I report on the testing of reporter strains. The aims of this chapter were to: -

1. Test whether the reporter constructs produce significantly different fluorescence when the reporter strains are exposed to either of two biocides known to induce *tolC*, *marR*, and *soxS*. This would confirm that the strains could be used as a tool to report response to other biocides.
2. Use any of the reporter strains that are shown to respond effectively to investigate the potential of NsARC to induce an adaptive resistance phenotype.

4.2 Results

4.2.1 Determining the effects of copper ammonium acetate on *BWtolC* and *BWsoxS* using quantitative fluorescence microscopy

Agents known to cause the adaptive response were used to test the reporter strains prior to using them with NsARC. The reporter strains were first characterised against the fungicide copper ammonium acetate ($C_2H_7CuNO_2$) because copper induces *tolC* transcription (Franke et al., 2003; Nishino et al., 2007). Copper also has been shown to

induce *marR* and *marR* has been shown to act as a copper sensor, possibly for when copper co-factors are lost from copper containing proteins (Hao et al., 2014). *soxS* has been shown to be induced up to threefold by copper exposure (Yamamoto & Ishihama, 2005).

The reporter strains each contained a plasmid with the open reading frame of the fluorescence gene *mScarlet* fused to each of the promoter regions from *tolC*, *marR*, and *soxS*.

At the concentrations used, copper had minimal effects on growth rates (Appendix: C). Each of the reporter strains was treated as follows: The strain was inoculated into two liquid cultures of LB broth, one of the liquid cultures also contained copper ammonium acetate, then both cultures were incubated for several hours. The cells from both cultures were then fixed. Fixed cells were visualised via fluorescence microscopy imaging and images were analysed to extract average brightness of the pixels of each individual cell. Averages of these brightness values were expressed as pixel brightness on an 8-bit image, 1-255. Fluorescence values for BWmarR were too low for analysis, and hence the analysis for these was discontinued.

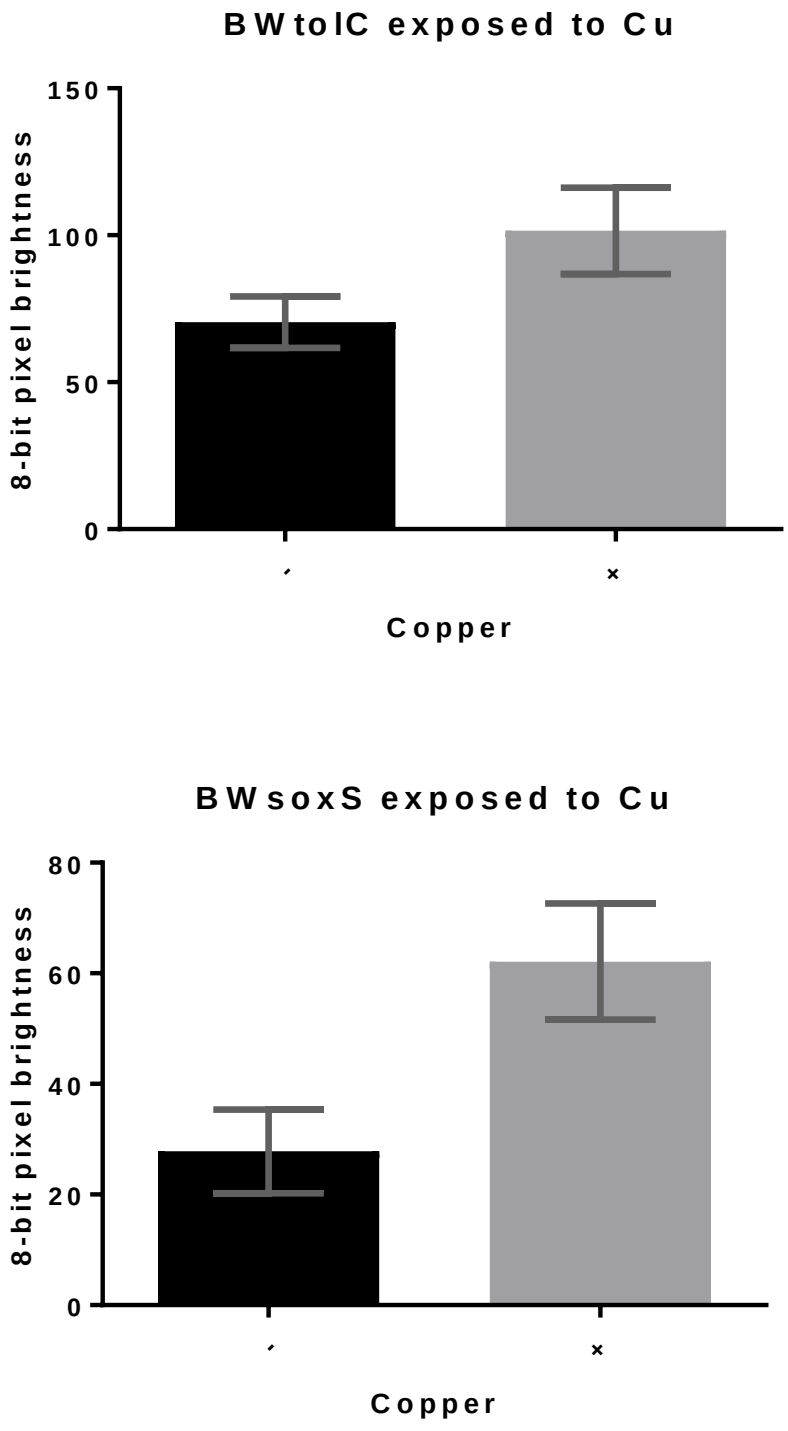


Figure 4. 2: Fluorescence in LB with and without copper ammonium acetate exposure for the strains BWtolC (Top) and BWsoxS (Bottom). Fluorescence is reported as 8-bit pixel brightness. Error bars are standard deviation (SD)

Table 4. 1: Mean fluorescence of strains unexposed and exposed to copper ammonium acetate. Fluorescence is reported as 8-bit pixel brightness. Differences between unexposed and exposed for strains BWtolC and BWsoxS tested via two-tailed paired t-test of 3 replicates each. Significance threshold is P-value < 0.05.

Strain	Mean pixel brightness (-)	Mean pixel brightness (+)	P value
BWtolC	70.47	101.5	0.0480
BWsoxS	27.81	62.12	0.0099

Copper ammonium acetate had a statistically significant effect on pixel brightness of BWtolC (Table 4.1, row 1), showing that the BWtolC strain was responding to copper. BWtolC copper exposure resulted in a 44% increase in mean pixel brightness compared to unexposed cells. Copper ammonium acetate had a statistically significant effect on pixel brightness of the BWsoxS strain (Table 4.1, row 2). Copper exposure resulted in a 123% increase in mean pixel brightness compared to unexposed. Both strains exhibited a fluorescence change that is indicative of efflux-associated gene expression changes.

4.2.2 Determining the effects of bright/dim colony selection and 2,4-D on *BWtolC, BWmarR,* and *BWsoxS* using quantitative fluorescence microscopy

Over the course of using the reporter strains I noticed a large amount of variation in fluorescence among individual colonies of the same strain (Figure 4.3).

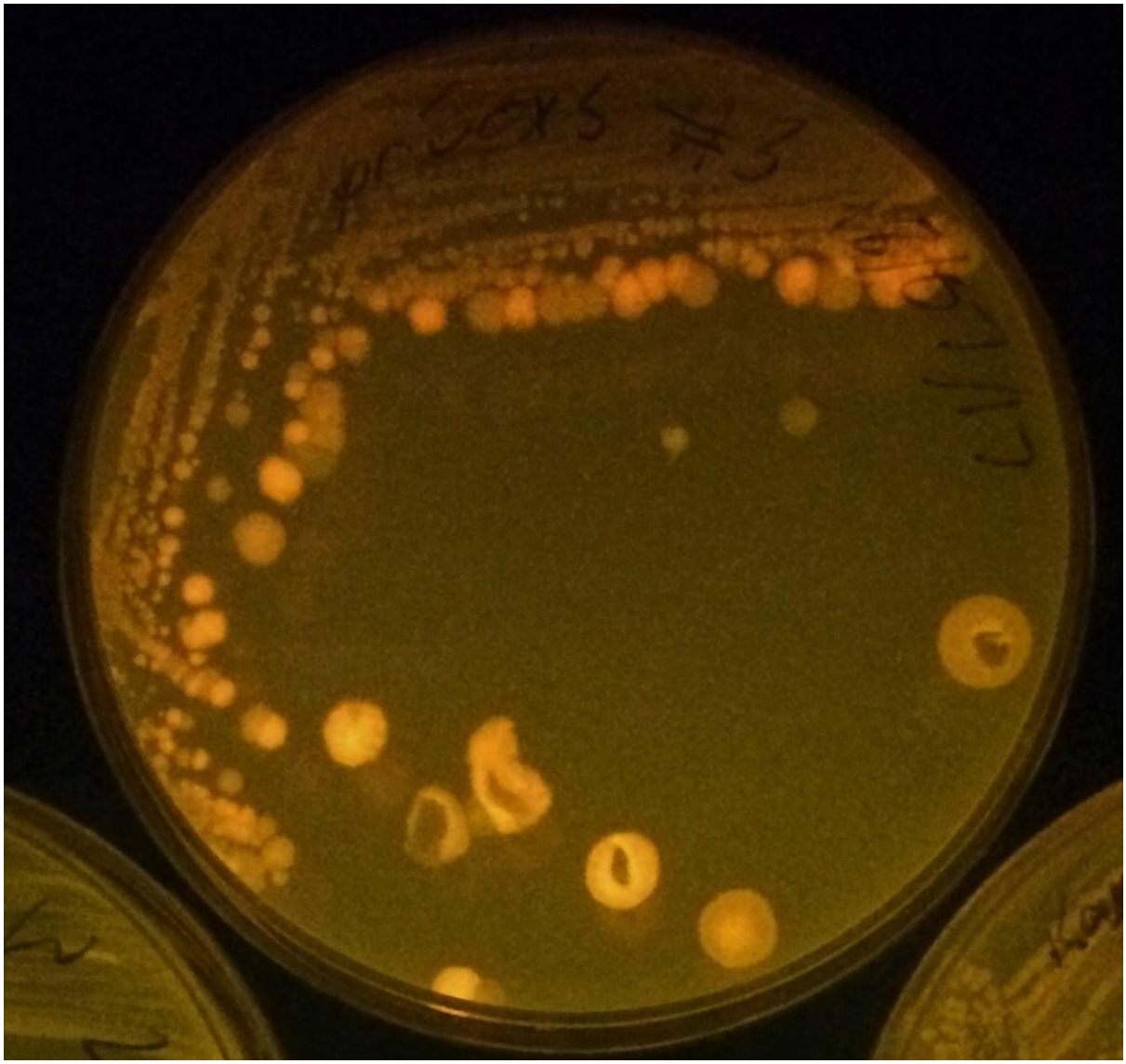


Figure 4. 3: Strain BWsoxS on 90 mm plate of LB agar with kanamycin (40 µg/ml). Illuminated from underneath by UV light. Variation in the brightness of individual colonies is visible.

This observation generated the hypothesis that this phenomenon is caused by varying rates

of gene expression. To determine if the variation was due to individual genetic differences or to random variation in gene expression, I tested whether the phenotypes bred true. To initially test this for each strain the bright and dim colonies were selected and re-streaked twice selecting for the brightest and dimmest strains. The results for the BWtolC strain can be seen in Figure 4.4.

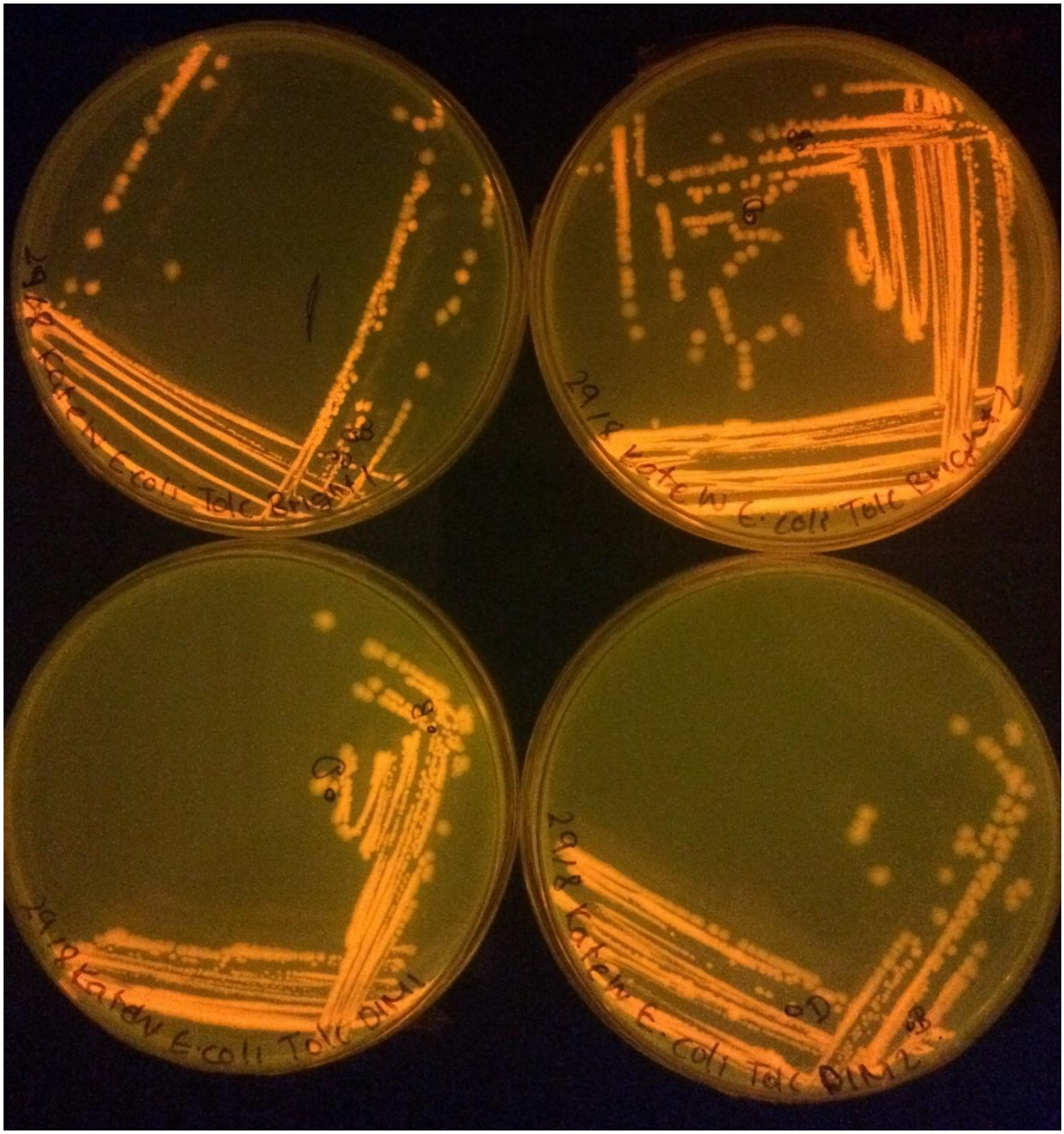


Figure 4. 4: Strain BWtolC on 90 mm plates of LB agar with kanamycin (40 µg/ml). Illuminated from underneath by UV light. Bright colonies restreaked on new plates (Top left and right). Dim colonies restreaked on new plates (Bottom left and right). Overall the colonies on the bright isolates appear brighter than those on the dim isolates. Variation in the brightness of individual colonies is still visible, especially on bright isolates.

For each of the original three reporter strains, four isolates were stored for later use.

Visual observation showed that the bright and dim strains did appear to be giving rise to colonies that were brighter or dimmer, but variation between colonies remained. This suggested that the strains were breeding true and that there was some genetic and heritable basis for the variation between colonies.

It also raised the following hypothesis: all isolates do not respond equally to an inducer. This means that the fluorescence difference between no exposure and exposure to an inducer could be more for some than others. Those with the largest gap in fluorescence between noninduced and induced would be most suitable for use as a reporter strain and future testing of NsARC.

To investigate the hypothesis of unequal fluorescence induction between isolates and to continue the characterisation of the fluorescent strains for future use, the bright and dim isolates were each treated as a new strain. Each of these bright and dim isolates was used in to test the effects of 2,4-D on reporter strains and treated as a separate strain.

The biocide used in this experiment was 2,4-Dichlorophenoxyacetic acid (2,4-D). 2,4-D was used to provide further evidence that strains act as anticipated. Previous research has shown that 2,4-D exposure increases expression of *tolC* in *Pseudomonas putida* and it has been used as an inducer before (Benndorf, Thiersch, Loffhagen, Kunath, & Harms, 2006). Induction of the *marRAB* locus has been studied in a similar fashion through the use of 2,4-D and similar chemicals (Alekshun & Levy, 1999; Balagué & Véscovi, 2001). MicF post transcriptionally controls expression of the outer membrane porin gene *ompF* (Delihas & Forst, 2001) and increased expression of *micF* has been shown with exposure to 2,4-D (Ben-Israel, Ben-Israel, & Ulitzur, 1998), *micF* transcription can be activated by SoxS (Z. Li & Demple, 1994). Given the low brightness of the BWmarR strain in the copper experiment, only the bright strains from BWmarR were used and analysed in this experiment.

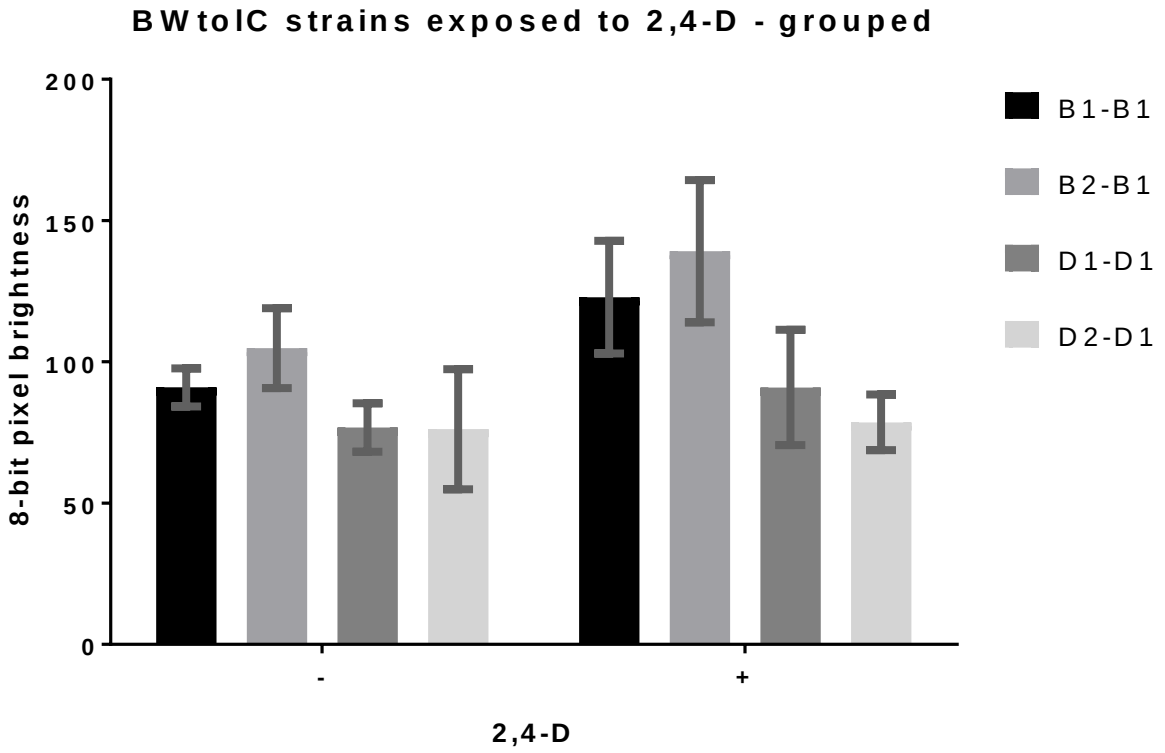


Figure 4. 5: BWtolC bright and dim strains exposed and unexposed to 2,4-D. Fluorescence values reported as 8-bit pixel brightness. Error bars are standard deviations.

Table 4. 2: Results of two-way ANOVA analysis of BWtolC strains. Factors are; 2,4-D exposure (exposed and unexposed); and Strain (bright and dim strains selected for). Significance threshold is P-value < 0.05

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
INTERACTION	6.482	0.0930
2,4-D +/-	16.16	0.1424
STRAIN	48.29	<0.0001

Across all strains neither 2,4-D nor interaction between strain selection and 2,4-D exposure had a significant effect on pixel brightness values (Table 4.2, rows 1 and 2). This suggests 2,4-D does not influence all strains equally.

ANOVA analysis shows that strain is a significant factor (Table 4.2, row 3). This suggests that the strain selection had more of an effect on the fluorescence than did 2,4-D exposure. This provides further evidence that changes in brightness between strains is heritable and may therefore have a genetic basis.

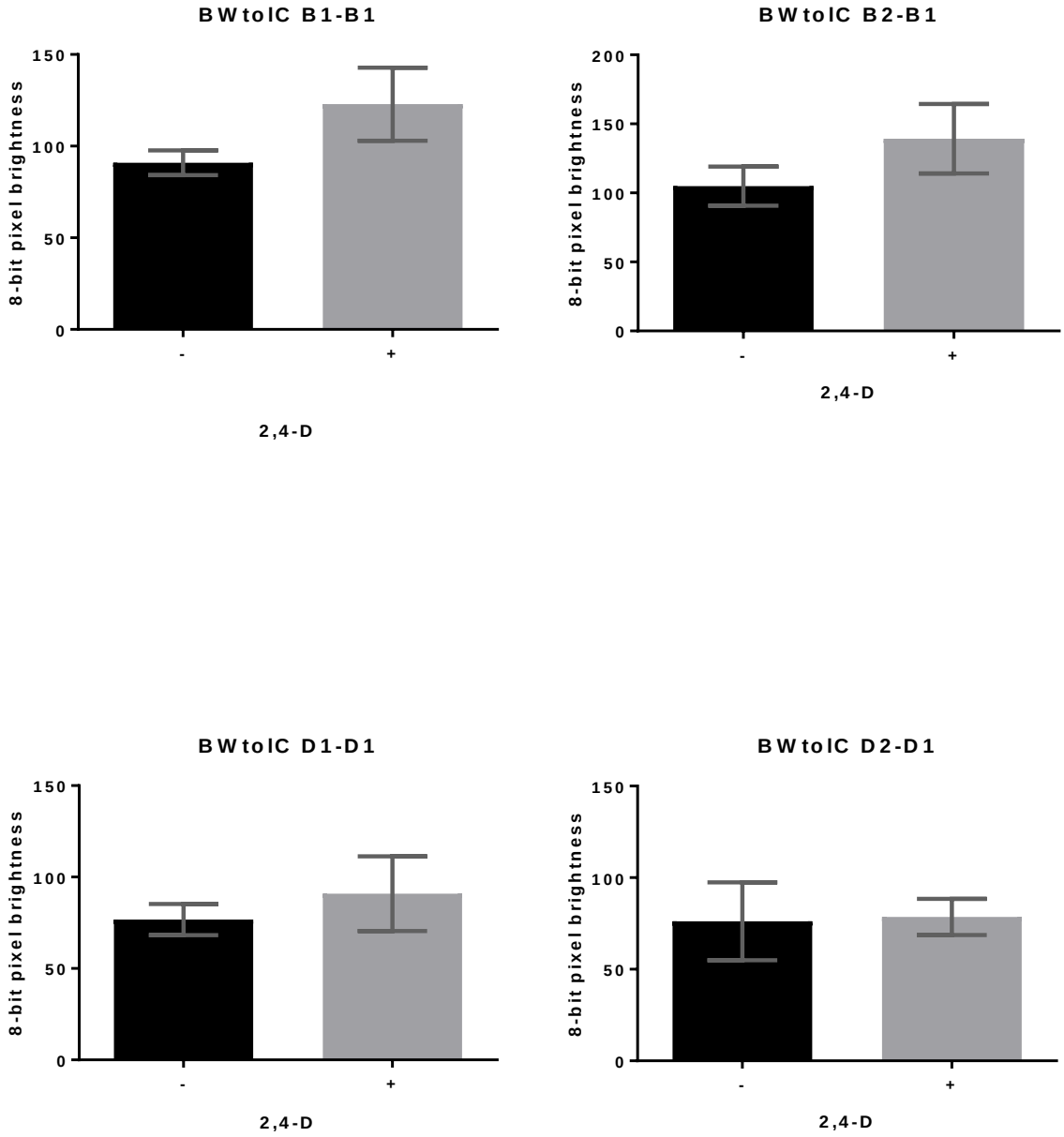


Figure 4. 6: Fluorescence in LB with and without 2,4-D exposure for the BWtolC strains B1-B1 (top left), B2-B1 (top right), D1-D1 (bottom left), and D2-D1 (bottom right). Fluorescence is reported as 8-bit pixel brightness. Error bars are standard deviation.

Table 4. 3: Mean fluorescence of BWtolC strains unexposed and exposed to 2,4-D. Fluorescence is reported as 8-bit pixel brightness. For each strain differences between unexposed and exposed treatments were tested via two-tailed paired t-test of 3 replicates each. Significance threshold is P-value < 0.05.

Origin Strain	Isolate	Mean pixel brightness (2,4-D-)	Mean pixel brightness (2,4-D+)	P value
BWtolC	B1-B1	90.97	122.9	0.0709
	B2-B1	104.9	139.2	0.0333
	D1-D1	76.75	90.91	0.1779
	D2-D1	76.17	78.6	0.7496

2,4-D treatment had no significant effect on the dim strains, D1-D1 and D2-D1 (Table 4.3, rows 3 and 4), their means showed approximately 18% and 3% increases, respectively. 2,4-D exposure on the bright strain, B1-B1, nonsignificant (Table 4.3, row 1) but a 35% increase in mean fluorescence warrants further investigation. 2,4-D treatment had a statistically significant effect on the bright strain, B2-B1 (Table 4.3, row 2), its showed a 33% increase in mean fluorescence. These analyses suggest that the bright strains of BWtolC respond to 2,4-D exposure more than the dim strains as would be expected. The bright strains may therefore be suitable for future testing with NsARC.

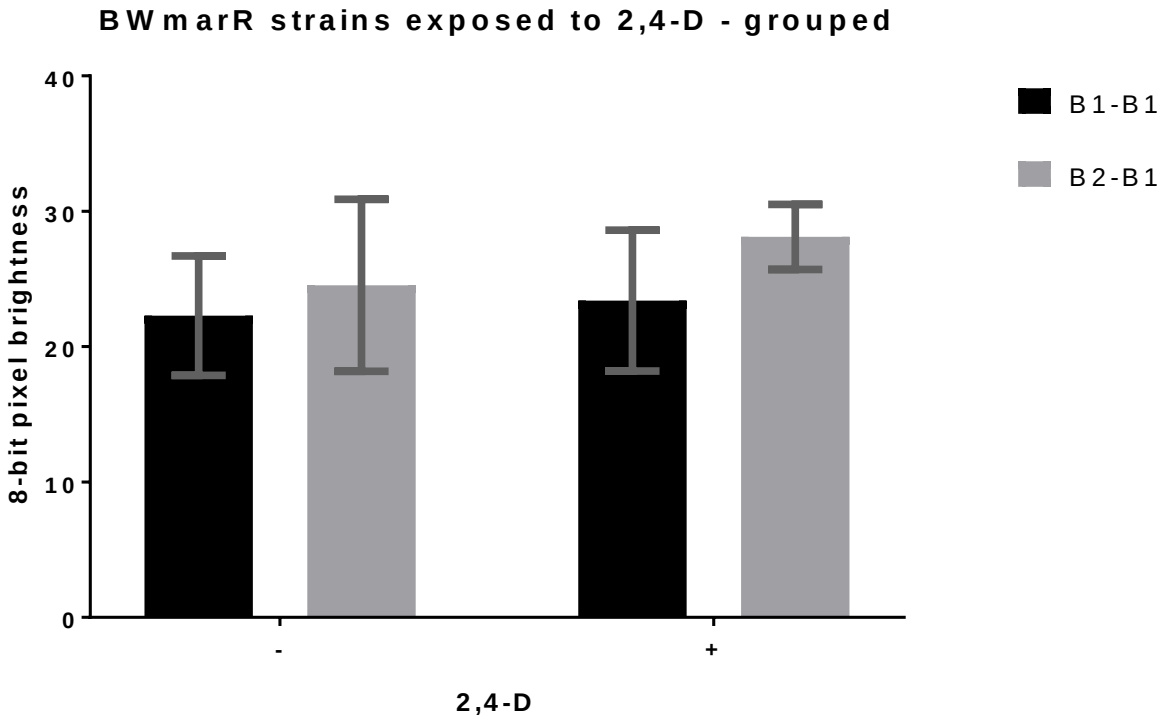
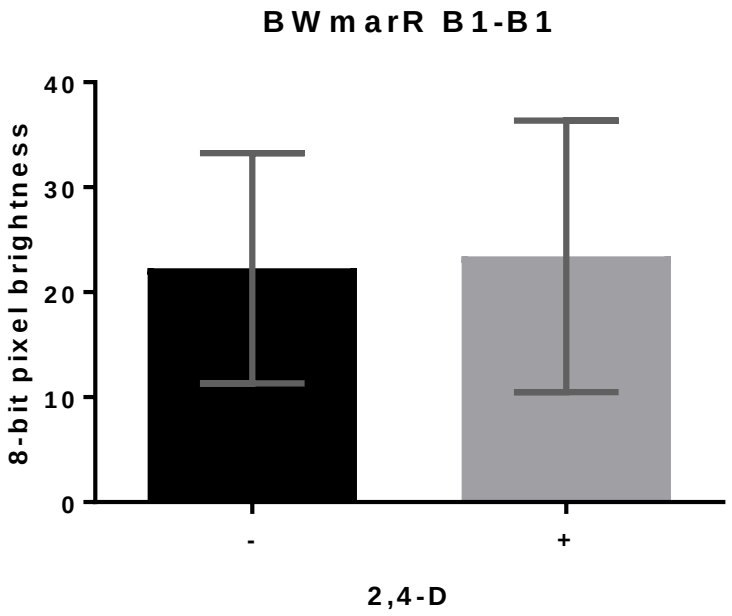


Figure 4. 7: BWmarR bright strains exposed and unexposed to 2,4-D. Fluorescence values reported as 8-bit pixel brightness. Error bars are standard deviations.

Table 4. 4: Results of two-way ANOVA analysis of BWmarR strains. Factors are; 2,4-D exposure (exposed and unexposed); and Strain (bright and dim strains selected for). Significance threshold is P-value < 0.05.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
INTERACTION	1.855	0.5772
2,4-D +/-	6.801	0.5248
STRAIN	14.96	0.1603

The exposure of BWmarR isolates to 2,4-D showed that across both strains neither 2,4-D nor strain selection nor interaction between the two had a significant effect on pixel brightness values (Table 4.4, rows 1, 2, and 3). This suggests that 2,4-D does not have an effect on *marR* expression in either strain. Strain selection was not shown to be a significant factor, this suggests that the strain selection regime had no effect on the fluorescence therefore the variation in brightness between strains and colonies may not be heritable in BWmarR.



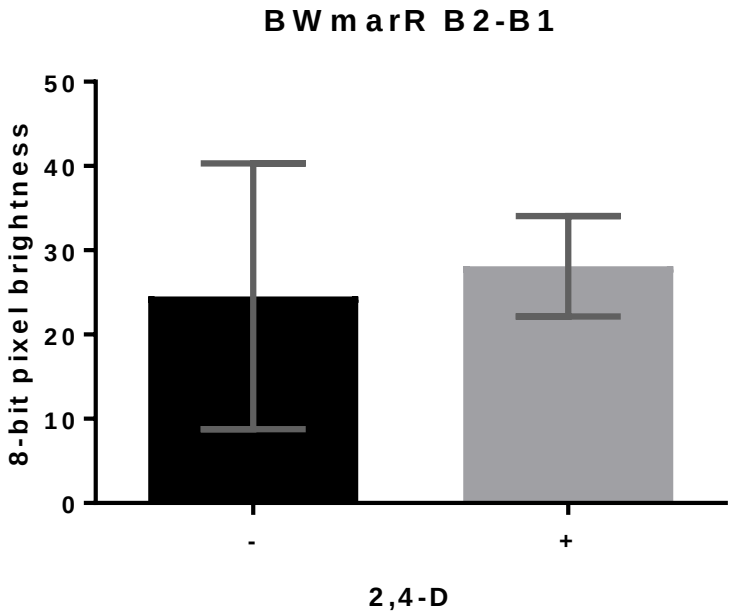


Figure 4. 8: Fluorescence in LB with and without 2,4-D exposure for the BWmarR strains B1-B1 (top), B2-B1 (bottom). Fluorescence is reported as 8-bit pixel brightness. Error bars are standard deviation.

Table 4. 5: Mean fluorescence of BWmarR strains unexposed and exposed to 2,4-D. Fluorescence is reported as 8-bit pixel brightness. For each strain differences between unexposed and exposed treatments were tested via two-tailed paired t-test of 3 replicates each. Significance threshold is P-value < 0.05.

Origin Strain	Isolate	Mean pixel brightness (2,4-D-)	Mean pixel brightness (2,4-D+)	P value
BWmarR	B1-B1	22.28	23.4	0.5027
	B2-B1	24.54	28.11	0.3345

Exposure of the bright strains, B1-B1 and B2-B1 to 2,4-D had no significant effect on pixel brightness (Table 4.8, rows 1 and 2), their means showed approximately 5% and 15% increases respectively. This supports the result of the ANOVA analysis on the two BWmarR strains together, showing that there appears to be no significant interaction between 2,4-D and changes in expression of MarR or the difference in starting strains were already too low.

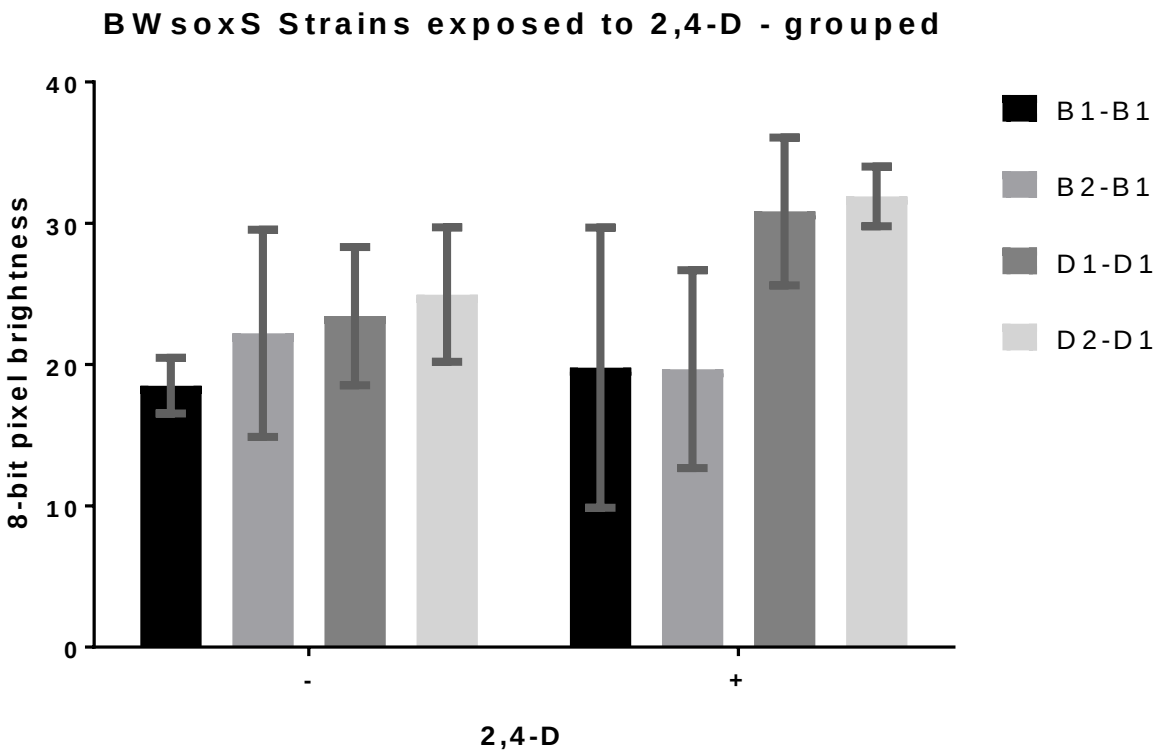


Figure 4. 9: BWsoxS bright and dim strains exposed and unexposed to 2,4-D. Fluorescence values reported as 8-bit pixel brightness. Error bars are standard deviations.

Table 4. 6: Results of two-way ANOVA analysis of BWsoxS strains. Factors are; 2,4-D exposure (exposed and unexposed); and Strain (bright and dim strains selected for). Significance threshold is P-value < 0.05.

SOURCE OF VARIATION	% OF TOTAL VARIATION	P VALUE
INTERACTION	9.259	0.4337
2,4-D +/-	5.801	0.1970
STRAIN	33.72	0.0397

Across all BWsoxS strains neither 2,4-D exposure nor interaction between strain selection and 2,4-D exposure had a significant effect on pixel brightness values (Table 4.6, rows 1 and 2). This suggests 2,4-D does not have an effect on all strains equally. Strain selection is a statistically significant factor (Table 4.6, row 3), this suggests that the strain selection had more of an effect on the pixel brightness values than the 2,4-D exposure. This provides further evidence that changes in brightness between strains is heritable, and therefore may have a genetic basis.

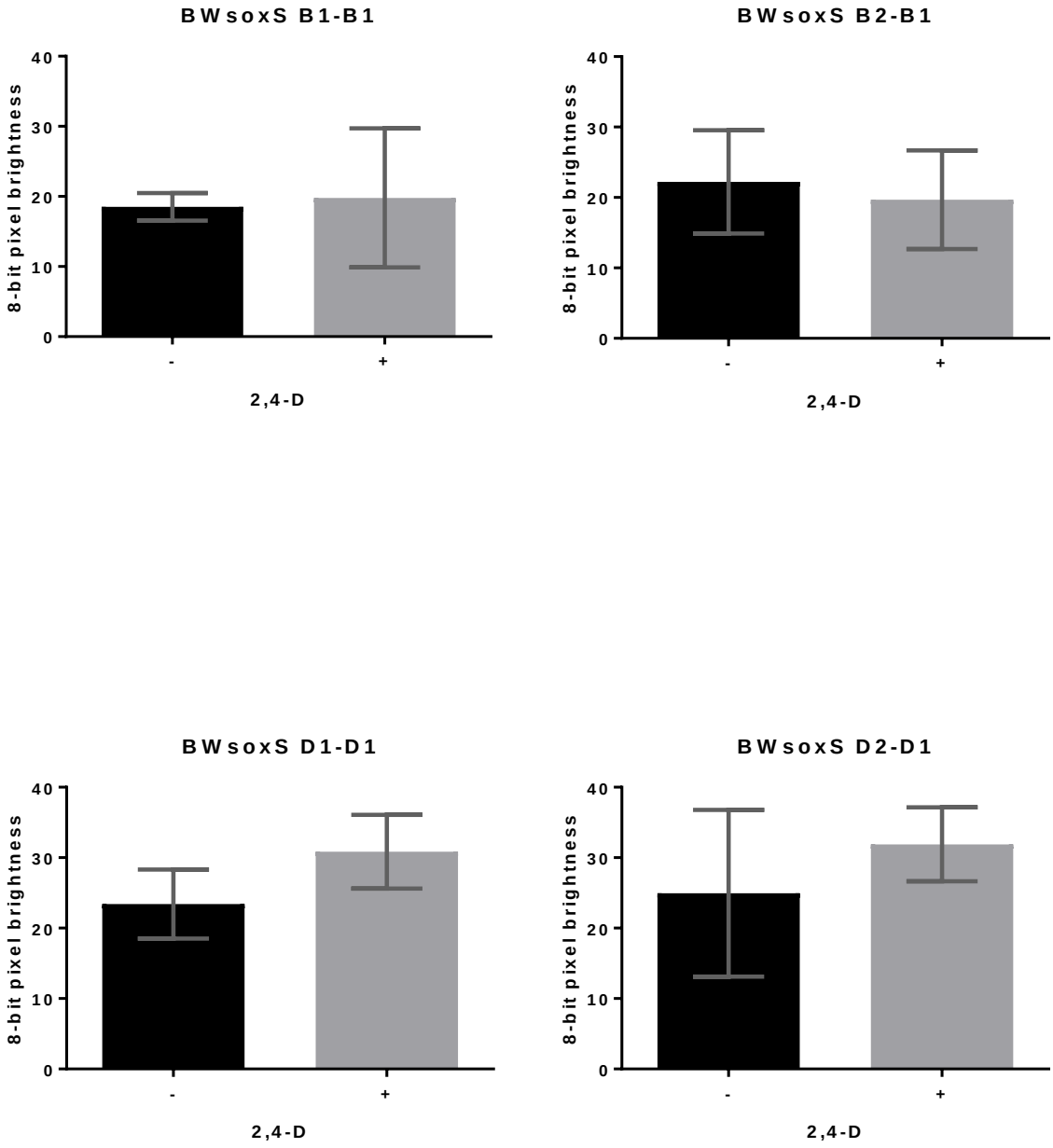


Figure 4. 10: Fluorescence in LB with and without 2,4-D exposure for the BWsoxS strains B1-B1 (top left), B2-B1 (top right), D1-D1 (bottom left), and D2-D1 (bottom right). Fluorescence is reported as 8-bit pixel brightness. Error bars are standard deviation.

Table 4. 7: Mean fluorescence of BWsoxS strains unexposed and exposed to 2,4-D. Fluorescence is reported as 8-bit pixel brightness. For each strain differences between unexposed and exposed treatments were tested via two-tailed paired t-test of 3 replicates each. Significance threshold is P-value < 0.05.

Origin Strain	Isolate	Mean pixel brightness (-)	Mean pixel brightness (+)	P value
BWsoxS	B1-B1	18.51	19.79	0.8689
	B2-B1	22.21	19.67	0.6804
	D1-D1	23.43	30.84	0.3037
	D2-D1	24.95	31.9	0.0465

2,4-D exposure on the bright strains, B1-B1 and B2-B1 had no significant effect (Table 4.7, rows 1 and 2). The mean of B1-B1 showed approximately 7% increase while the mean of B2-B1 actually showed a decrease in mean pixel brightness of 11%. 2,4-D exposure on the dim strain, D1-D1, also had no significant change in pixel brightness (Table 4.7, row 3), with a 31% increase in mean pixel brightness. 2,4-D exposure on the dim strain, D2-D1, was significant with a P-value of 0.0465 (Table 4.7, row 4), and a 28% increase in mean pixel brightness. These analyses suggest that the strain D2-D1 of BWsoxS may be suitable for future analysis of NsARC.

4.2.3 Determining the effects of NsARC on *BWtolC* using quantitative fluorescence microscopy

This section describes a preliminary experiment to investigate the possible effects of NsARC on fluorescence expression in the *E. coli* reporter strain BWtolC B2-B1. The experiment was carried out as described in section 2.1.10. Given the limited supply of NsARC samples it was only possible to perform one replicate of this experiment, so no statistical analysis was possible.

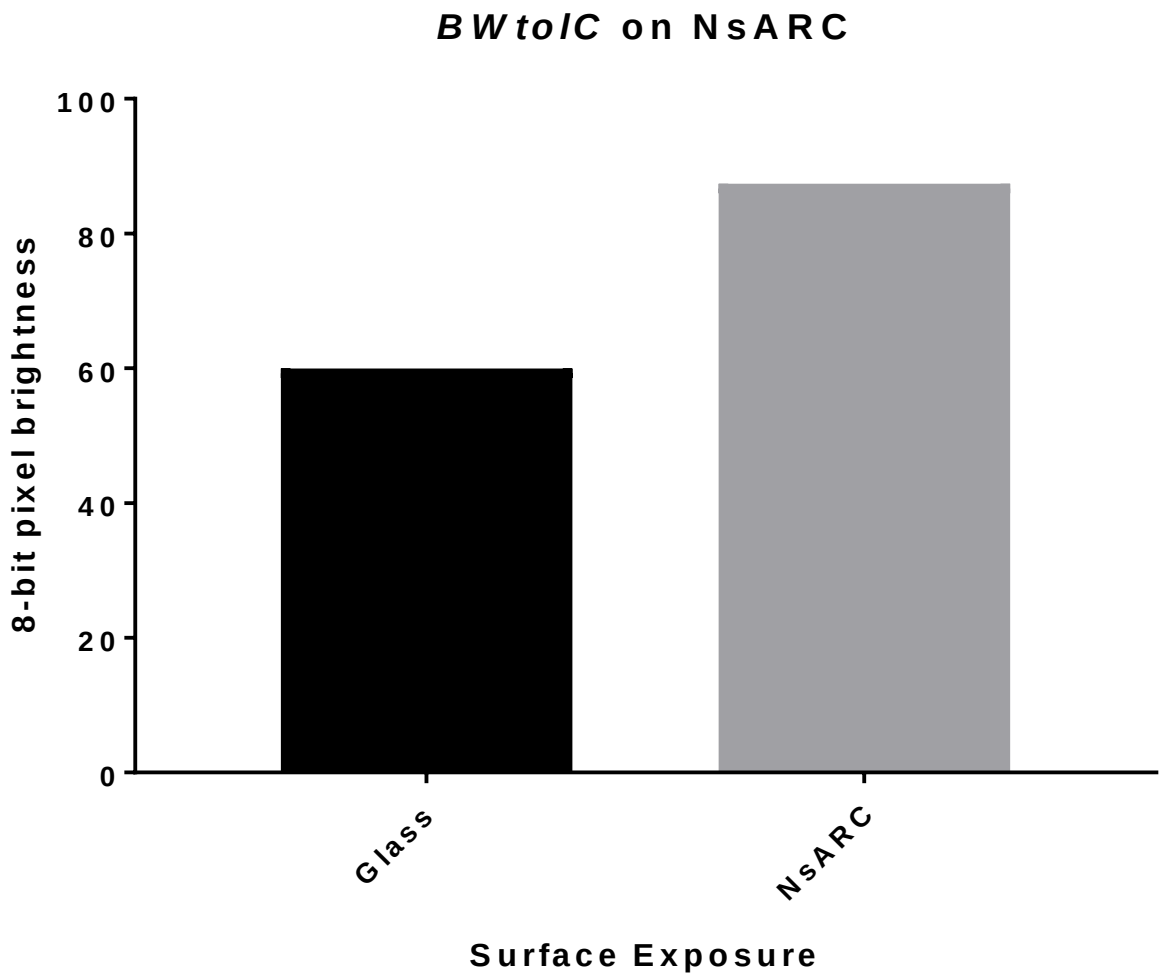


Figure 4. 11: Average fluorescence of reporter cells of BWtolC B2-B1 upon exposure to glass and NsARC. Fluorescence is reported at 8-bit pixel brightness.

Figure 4.11 shows that the average 8-bit pixel brightness for NsARC is higher than glass. The brightness value for glass exposure was 59.98, while the brightness value for NsARC exposure was 87.37. This shows that replication of this experiment is warranted as there may be a statistically significant difference between exposures.

With the exposure of BWtolC to copper ammonium acetate the average percentage increase was 44%, while the average change in pixel brightness when BWtolC B2-B1 was exposed to 2,4-D was only 33%, but both of these changes were shown to be statistically significant. NsARC exposure showed 45% higher brightness than unexposed. This indicates

that replication of the experiment may well show that exposure of BWtolC to NsARC causes a statistically significant change in average cell fluorescence.

4.3 Discussion

BWtolC strains exposed to copper ammonium acetate fluoresced more than unexposed controls. This suggested that the reporter strains were functioning as expected when exposed to copper.

Exposure of the strain BWmarR to copper ammonium acetate did not result in images where the fluorescence values were quantifiable. This was due to a low exposure time and the reliance on a direct image contrast (DIC) objective in the fluorescence microscope at the time. Use of the DIC objective in fluorescence microscopy resulted in being unable to analyse the individual cell fluorescence values effectively because the process of picking them out from the background was unreliable at these low fluorescence levels. A higher image exposure time when imaging and the use of the phase contrast objective (which was obtained for the fluorescence microscope during the latter part of this research project) means cells of BWmarR could be analysed and a quantitative result generated from that. It was expected that changes in *marR* expression would be significant as past research on *marR* expression when exposed to different biocides has shown significant increases in RNA expression levels (Kurenbach et al., 2017).

Copper ammonium acetate caused a significant increase in BWsoxS fluorescence compared to unexposed controls. This suggests that the reporter strain was functioning as expected when exposed to copper.

The fluorescence response of the strains BWtolC and BWsoxS to a known biocide being in line with that of *tolC* and *soxS* expression changes in previous research indicate that BWtolC and BWsoxS strains may be suitable for future use testing biocides.

However, increases in fluorescence could also be due to minor differences in growth rates between strains exposed or not to copper ammonium acetate that were observed during experimentation (Appendix: C). The reporter strains were investigated for differences in growth rates due to copper exposure. The biggest difference observed between exposed and unexposed strains was that exposed strains were at most 10-fold lower in concentration. This was deemed marginal at the time, but it may account for an accumulation of fluorescent protein, as cells have a tendency to accumulate efflux pumps and even increase expression of efflux pump genes during slow growth rates (Rand, Danby, Greenway, & England, 2002).

4.3.2 2,4-D induced changes in efflux pump expression in selected reporter strain isolates

I used a biocide with known effects on the adaptive response to further characterise the reporter strains. Like copper, 2,4-D exposure has been shown to be associated with the induction of the genes *tolC*, *marR*, and *soxS* (Alekshun & Levy, 2004; Ben-Israel et al., 1998; Benndorf et al., 2006; Z. Li & Demple, 1994).

During early experimentation with the reporter strains I observed a large amount of variation in fluorescence brightness between colonies. The initial hypothesis was that this was caused by variation in gene expression or an impure culture, but this hypothesis was disproven through serial culture of individual colonies on plates. While the average brightness of colonies derived from a bright colony were brighter, colony to colony differences were still observable.

In order to test if the differences between selected strains were statistically significant, each was tested by exposure to 2,4-D. 2,4-D exposure was also used to test the hypothesis that some selected strains are better suited as reporter strains than others.

There was a statistically significant effect on fluorescence values from selecting for bright and dim strains in both the BWtolC strains and BWsoxS strains. This showed that the differences between strains were heritable, measurable and statistically verifiable. This suggests that the differences between strains are genetic in origin.

The plasmid copy number could be the source of variations in strain brightness. Changes in copy number could be due to mutations in plasmid copy number regulation. The plasmids in the reporter strains are based on pFru97, this plasmid uses *pBBR1 oriV* as its origin of replication (Remus-Emsermann, Gisler, & Drissner, 2016) and is present in a medium copy number (10-20) per cell (Khan, Gaines, Roop, & Farrand, 2008; Kovach et al., 1995). Mutations causing variation in plasmid copy number have been well researched in the past (Lopilato, Bortner, & Beckwith, 1986; Tao, Jackson, Rouvière, & Cheng, 2005; Weisblum, Graham, Gryczan, & Dubnau, 1979). Despite mutations in plasmid copy number control being a possibility, it seems unlikely there would be such a high rate of mutation within these genes. Mutation of genes controlling plasmid copy number does not appear to explain the observation of different brightness levels on a colony by colony basis. An experiment to perform to check for mutations in the plasmid would be to isolate the plasmid from a dim strain of the bacteria, then put it into the same base bacterial strain (in this case BW25113) and observe if dim colonies proliferate, if so this would suggest a mutation in the plasmid. The isolated plasmid could also be sequenced. An experiment could be performed to see if there was a correlation between plasmid copy number and strain brightness. It is possible to use the antibiotic resistance genes (*nptII* and *cat*) on the plasmid (pFru97) to quantify plasmid copy number, this has been done with *E. coli* using the *cat* gene and *bla* genes (Klotsky & Schwartz, 1987). Methods that rely on inference of plasmid number from enzyme activity are imprecise. However, only qualitive results are

required rather than quantitative because the only information needed is whether or not there is more plasmid copies in brighter strains. One would only need to investigate if the bright strains have a higher plasmid copy number than the dim strains. If this were the case, the difference in fluorescence would have been due to a difference in plasmid copy number.

Another possibility is a high rate of mutation in the plasmid and therefore the promoter region of the fluorescent protein. Again, the mutation rate seems high and the probability of the same mutation occurring in the same place in many different cells is vanishingly small. If plasmid copy number is found to be stable between strains and sequencing shows no difference, the possibility of changes in the plasmid can be tested by testing if brighter strains display higher antibiotic resistance. If they do, then it is likely that the brighter strains have more expression of efflux pump genes as well as the fluorescent protein gene. This would suggest that the changes in brightness are due to changes in one or more efflux pump transcriptional regulators rather than changes in the cloned promoters. Again, the likelihood of such a high mutation rate in promoter regions specific to efflux expression is vanishingly small but can be initially investigated by sequencing these regions and checking for promoter mutations.

Although the cause for it is not immediately obvious there is a heritable and statistically significant difference between different selected strains for both BWtolC and BWsoxS. BWmarR shows no significant difference between the strains tested but this may be due to only bright strains being analysed for their response to 2,4-D exposure (Table 4.8).

Each bright and dim strain was also analysed for statistical differences from exposure to 2,4-D via two-tailed T-Tests.

Table 4. 8: Results summary of significance testing of bright and dim strains exposed and unexposed to 2,4-D. Significance was tested via paired two-tailed t-tests. Significance threshold P-value < 0.05. Significant (+), nonsignificant (-), Strain not tested (N/A).

	Origin Strain		
	BWtolC	BWmarR	BWsoxS
B1-B1	-	-	-
B2-B1	+	-	-
D1-D1	-	N/A	-
D2-D1	-	N/A	+

These results showed that among BWtolC strains, B2-B1 showed a significant change in brightness from 2,4-D exposure. Strain B1-B1 was borderline nonsignificant and may warrant further investigation. BWsoxS strain D2-D1, showed a significant response from 2,4-D exposure. No BWmarR strains showed a significant response from 2,4-D exposure. New strains should be constructed for that gene. Variation between strains due to strain selection may account for the differences in responses to 2,4-D. The differences between strains may result in minimal changes in fluorescence in some instances, this could be the case if strain differences are caused by high plasmid copy number, as overall signal for activation of efflux promoters could be divided among the numerous plasmids, thus minimising the effect. The problem in some cases may also be that 2,4-D is not a strong inducer of adaptive resistance. 2,4-D has been used as an inducer of adaptive resistance. Kurenbach et al., (2015) reported that the response of adaptive resistance from 2,4-D exposure at some antibiotic concentrations follow trends of changed adaptive resistance to the antibiotic but was still statistically nonsignificant; it may be the same here. In the case of

BWtolC strains 2,4-D exposure consistently resulted in a higher average fluorescence value, despite showing statistical significance in only one strain.

These results show there is a heritable variability with a high rate of variation among individual colonies of the reporter strains and some are more useful as reporter strains than others when tested against biocides. Given the results of experimentation to characterise these strains, only BWtolC B2-B1 and BWsoxS D2-D1 appear suitable for future investigation of the effects of NsARC on efflux pump expression. BWmarR is unsuitable for further testing. It maybe that the initial design of the strain was incorrect with an insufficient region of the promoter selected, or it may be due to errors during the making of the plasmid construct. The promoter region may be inverted adjacent to the fluorescent gene, but still allowing low level expression of the fluorescent gene. But further characterisation of the BWmarR strains is required to ascertain if it is indicative of *marR* expression.

With the current level of characterisation of the BWtolC and BWsoxS strains and without explanation for the high variation between individual colonies when viewed on the plate it is recommended that they can only be used to offer an indication of whether or not a given biocide is inducing certain efflux pump expression, not quantifying how much said expression is induced.

4.3.3 Initial results indicate that NsARC induces changes in *tolC* expression

The use of strains reporting on efflux activity was devised as a method for investigating the potential effects of NsARC on efflux activity. This investigation was proposed as part of a responsible approach to the introduction of a new material; investigating it for potential effects on adaptive antibiotic resistance. Small changes in resistance can enable a pathogen to survive at higher concentrations of antibiotic and thus make larger changes in antibiotic resistance via mutation or gene acquisition more likely to

occur (Fernández et al., 2011). It is this potential for NsARC that will be investigated in research to come.

Preliminary testing of the effects of NsARC exposure on the reporter strain BWtolC B2-B1 was performed using ambient lighting levels and without a control in the dark. It was hypothesised that the antibacterial effects that have been shown by NsARC regardless of light levels should be tested. The decision to only use one light treatment was also made due to limitations in the supply of NsARC test pieces and consequently only one experiment was performed. But in this one replicate, NsARC exposure appears to increase average pixel brightness by approximately 45% and indicates the potential for a significant response.

Any NsARC induced increase in cell fluorescence may be due to oxidative activity of the material. A theory for the antibacterial activity of NsARC regardless of light levels was that it may generate carbon-centred oxidative activity (Fenoglio Ivana et al., 2009). The exposure of cells to oxidative species could induce *soxR*, which in turn would allow transcription of *soxS* (Nunoshiba et al., 1992), which in turn would cause transcription of *tolC* by interaction with the *tolC* promoter and induce the fluorescence gene also under the control of a *tolC* promoter (A. Zhang et al., 2008).

5.0 Summary and Future Direction

This study aimed to explore the characteristics of NsARC, determining its antimicrobial activity under various conditions. NsARC was tested for antimicrobial activity while exposed to UV or visible light of different intensities.

This study also aimed to establish appropriate methodologies to test whether NsARC could contribute to the evolution of antibiotic resistance, in particular adaptive multi-drug resistance. Reporter strains were constructed and characterised by their responses to biocides previously used to induce changes in efflux associated gene expression.

Hospital acquired infections are a constant threat to immunocompromised individuals and are becoming even more dire as antibiotic resistance of pathogens increases. It was estimated in the USA in 2010 that hospital acquired infections increases health costs by an estimated $16.6 billion annually and this will only get worse without intervention (Hassan et al., 2010).

Infection prevention is one of the primary concerns when dealing with immunocompromised individuals and success has been seen with ever more tightening standards of hospital and patient sanitation. It was shown that It was shown that daily bathing of patients chlorhexidine impregnated washcloths significantly decreased the rate of nosocomial infections during a hospital trial (Climo et al., 2013). But this method still allows for the spread of pathogens from patients yet to be cleaned and to patients just cleaned. The introduction of antimicrobial surfaces to hospital environments has been used in the past to have the effect of reducing the microbial burden in hospital environments, this effect has been well studied with copper surfaces (Karpanen et al., 2012; Mehtar et al.,

2008; Schmidt et al., 2012). But given the costs of copper and outfitting entire hospitals it seems unlikely that this is a viable option.

The introduction of a cheap antimicrobial coating material would be extremely welcome to decrease the rate of pathogen transmission via touch surfaces and decrease the microbial burden in hospitals. TiO_2 has been heavily researched for this use and others due to photocatalytic properties, but much of this property is limited by primarily working in UV light (Foster et al., 2011). There has been a great deal of research recently on novel formulations of TiO_2 coatings that are active in visible light (Pelaez et al., 2012) as well as just UV and NsARC is one of these coatings. As a novel formulation NsARC required testing to establish whether it had any antimicrobial properties and whether or not they were photocatalytic in nature in both UV and visible light.

My results show that NsARC does have antimicrobial properties. These properties are observable in darkness, UV light, and visible light, but are increased by UV or high intensity visible light. The fact that the antimicrobial activity is enhanced by some forms of light shows that at least some of the effect is photocatalytic, but the antimicrobial activity in darkness indicates that photocatalysis is not responsible for the entirety of the effect. One theory is that the carbon in the NsARC formulation is causing carbon-centred production of reactive oxygen species in the dark, this has been shown to occur with carbon-based materials interacting with TiO_2 by cleavage of the C-H bond (Fenoglio Ivana et al., 2009), it could be possible to test this by annealing NsARC in air to allow carbon to be removed via oxidation but leave the morphology unchanged. What I expect to see would be a decrease in the activity of NsARC under dark conditions.

Another question that was raised was the decreased effectiveness of NsARC pieces upon reuse. It appeared to be caused by the steel underneath corroding when in contact

with a highly saline solution and a large concentration of reactive oxygen species. A solution that was proposed was putting the NsARC coating on different materials, such as glass. This technique has been attempted and is has been reported to show more potential for being reusable.

The entirely of this thesis was performed using NsARC material produced under the uniform conditions to allow for results to be comparable and consistent, but Koti Technologies was consistently performing research and produced NsARC materials with different production conditions, such as different annealing temperatures and precursor solution concentrations. Initial research has shown that some of these variations of the NsARC formulation are much more photocatalytically active according to testing with dye degraded by oxidative activity. These other formulations of NsARC will require testing for antimicrobial activity and their effects on reporter strains.

Aside from the potential usefulness of NsARC for its antimicrobial properties, it was deemed important by Koti Technologies to consider some of the potential negative effects NsARC may have. NsARC is a biocidal material which might, like other biocidal materials unrelated to antibiotics induce higher levels of antibiotic resistance (Kurenbach et al., 2015a). If NsARC has the potential to be associated with higher levels of antibiotic resistance then it is prudent to know before widespread use, so that its use can be informed and hopefully less harmful. The reporter strains were designed to report on changes in expression of efflux pump associated genes, efflux gene expression being one of the primary mechanisms of adaptive resistance (Fernández & Hancock, 2012). The reporter strains were designed and constructed to report on the activity of 3 genes previously associated with adaptive efflux response: *tolC, marR,* and *soxS.* Prior to their use on NsARC the strains needed to be characterised to ensure that they were useable as reporters of adaptive

resistance expression changes. The reporter strains were characterised using two different biocides that had been used previously to induce efflux pump activity, copper ammonium acetate (Franke et al., 2003; Nishino et al., 2007; Yamamoto & Ishihama, 2005), and 2,4-D (Alekshun & Levy, 1999; Balagué & Véscovi, 2001; Kurenbach et al., 2015a). Bacterial fluorescence was analysed by fluorescence microscopy and image analysis.

The results from exposure to copper ammonium acetate showed that the strain BWtolC responded as expected of a reporter of *tolC* expression by statistically significantly increasing in fluorescence brightness. BWmarR experiments suffered from technical problems which left the results unusable. BWsoxS responded to copper exposure as expected of a reporter of *soxS* expression by statistically significantly increasing in fluorescence brightness.

Over the course of testing the reporter strains it was noticed that there appeared to be a large amount of variation in fluorescence brightness between colonies. Initial testing indicated that the traits of bright and dim were partially heritable, but variance remained between colonies. In order to test if the differences between bright and dim strains were significant and if some might function better than others as reporter strains, each strain was exposed to 2,4-D and differences between exposed and unexposed were measured by fluorescence microscopy.

Results showed that selection for bright and dim strains had a significant effect on fluorescence in the cases of both BWtolC derived strains and BWsoxS derived strains. However, only two of the strains showed significant changes in fluorescence from 2,4-D exposure, one was BWtolC B2-B1 and the other was BWsoxS D2-D1. These strains were assessed as being suitable for future investigation of the effect of NsARC on adaptive resistance associated genes.

A preliminary experiment was performed by exposing BWtolC B2-B1 to NsARC and to a glass control. Results indicated that the culture exposed to NsARC had a much higher average fluorescence than the culture exposed to glass. This suggests that NsARC exposure has the effect of inducing adaptive resistance responses in *E. coli*, but further replication of the experiment is needed to establish statistical significance. The construction and limited characterisation of the reporter strains done in this thesis forms a solid foundation of methodology that will be carried forward onto future research on NsARC and other biocidal materials that also may induce adaptive resistance gene expression.

If none of the reporter strains from BWmarR or BWsoxS are induced by NsARC exposure and replicates show that BWtolC B2-B1 is statistically significantly induced by NsARC, then it may be useful to build more reporter strains to investigate the potential mechanisms by which BWtolC is induced. A current hypothesis is that BWtolC fluorescence changes may be induced by *soxS* due to *soxRS* system acting as a sensor for oxidative stress (Nunoshiba et al., 1992; A. Zhang et al., 2008). NsARC antimicrobial activity was most likely being caused by oxidative activity from photocatalysis to some extent. If *soxS* expression is induced, it would result in the increased *tolC* expression (A. Zhang et al., 2008).

The gene *rob* codes another transcriptional activator of *tolC*, similar to MarA and SoxS, and *rob* transcription is induced by the presence of organic solvents and heavy metal exposure (Kwon, Bennik, Demple, & Ellenberger, 2000; Rosenberg, Bertenthal, Nilles, Bertrand, & Nikaido, 2003). It could be that nanostructured materials interact with the *rob* gene or Rob transcription factor, so construction of a reporter strain to investigate the effects of NsARC on it may be warranted.

Further investigation into the bright and dim strains could be needed because the cause of different brightness levels is still not understood. Bright and dim strains have been shown

to have an effect on the inducibility of fluorescence by biocides, without further investigation it could mean that the fluorescence induction of these strains is unreliable. One hypothesis is that the variation in brightness is due to variations in copy number of the plasmid present, but this does not account for the heritability of the bright and dim phenotypic traits, as variation in plasmid copy number would be assumed to be relatively constant within a strain rather than varying between colonies. Variation in plasmid copy number could be measured by testing both bright and dim strains of reporter strains via qPCR (C. Lee, Kim, Shin, & Hwang, 2006). Others have quantified copy number by correlating the level of resistance to antibiotics granted by resistance genes with plasmid copy number. Chloramphenicol and kanamycin resistance genes of the plasmids could be used to quantify plasmid copy number (Klotsky & Schwartz, 1987). Another hypothesis to explain the heritable difference in strain fluorescence levels are mutations in the promoter regions controlling expression of the fluorescence genes on the plasmids. Any potential changes caused by mutation in these promoter regions could be investigated for by isolating the plasmid of the bright or dim strain and using it to again transform BW25113. If the bright or dim phenotype follows the plasmid, it would indicate changes in the plasmid. If it doesn't, then this would suggest changes in the genome or epigenetic changes causing different phenotypes. One final hypothesis is that the high rate of variation in bright and dim strains and their heritability may be caused by epigenetic factors, such as methylation patterns. This could be tested via the use of methylation sensitive and methylation non-sensitive isoschizomer restriction enzymes (Ko Hashimoto, Kokubun, Itoi, & Roach, 2007). Alternative, the isolated plasmid could be used to transform methylation deficient *E. coli,* e.g. *dam* and *dcm* deficient strains.

Research suggests that some antibiotic resistance genes can decrease susceptibility to photocatalytic materials. Tsai Ting-Mi et al., (2010) showed resistance to photocatalytic activity on vancomycin-resistant *E. faecalis*. The vancomycin susceptible strain displayed 2.4 times more susceptibility to inactivation by TiO_2 photocatalytic activity than the resistant counterpart. This suggests that cross resistance may exist between some antibiotic resistance genes and photocatalysis. If this cross resistance exists NsARC may have the effect of selecting for resistant strains of pathogens. It may be prudent to investigate if any antibiotic resistance genes also grant resistance to NsARC. This investigation could start with attempting to replicate the observations in Tsai Ting-Mi et al., 2010 by subjecting vancomycin-resistant *E. faecalis* and vancomycin-susceptible *E. faecalis* to antibacterial testing of NsARC as in section 2.1.6.

The transmission of pathogens via touch surfaces is and will continue to be a threat to human health. Any method that can lessen this problem is valuable. But any antimicrobial material that is introduced must be investigated fully for any negative effects it may have on the problem of antibiotic resistance as it is swiftly becoming a threat to the continued health of our species. The results presented in this thesis highlight that the novel photocatalytic material NsARC has antimicrobial properties under a range of conditions and may be very valuable for these properties. Furthermore, methodology was established by which NsARC could be investigated for the potential to induce higher levels of antibiotic resistance. Foresight such as this should be part of the genesis of any new technology and it is laudable that it was sought out in the case of NsARC. Any new technology should be fully examined for the benefits and costs it may have to society, therefore further work is needed on NsARC to fully elucidate what these may be.

6.0 References

Abraham, E. P., & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature*, *146*, 837.

Adams, L. K., Lyon, D. Y., & Alvarez, P. J. J. (2006). Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. *Water Research*, *40*(19), 3527–3532. https://doi.org/10.1016/j.watres.2006.08.004

Ahmad, H., Kamarudin, S. K., Minggu, L. J., & Kassim, M. (2015). Hydrogen from photo-catalytic water splitting process: A review. *Renewable and Sustainable Energy Reviews*, *43*, 599–610. https://doi.org/10.1016/j.rser.2014.10.101

Akhavan, O. (2009). Lasting antibacterial activities of Ag–TiO2/Ag/a-TiO2 nanocomposite thin film photocatalysts under solar light irradiation. *Journal of Colloid and Interface Science*, *336*(1), 117–124. https://doi.org/10.1016/j.jcis.2009.03.018

Alekshun, M. N., & Levy, S. B. (1999). The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends in Microbiology*, *7*(10), 410–413. https://doi.org/10.1016/S0966-842X(99)01589-9

Alekshun, M. N., & Levy, S. B. (2004). The Escherichia coli mar locus-Antibiotic resistance and more. *ASM News-American Society for Microbiology*, *70*(10), 451–456.

Alexander, J. W. (2009). History of the Medical Use of Silver. *Surgical Infections*, *10*(3), 289–292. https://doi.org/10.1089/sur.2008.9941

Amábile-Cuevas, C. (2016). Society must seize control of the antibiotics crisis. *Nature News*, *533*(7604), 439. https://doi.org/10.1038/533439a

Antibiotic Resistance Threats in the United States, 2013 | Antibiotic/Antimicrobial Resistance | CDC. (n.d.). Retrieved January 29, 2018, from https://www.cdc.gov/drugresistance/threat-report-2013/

Ariza, R. R., Cohen, S. P., Bachhawat, N., Levy, S. B., & Demple, B. (1994). Repressor mutations in the marRAB operon that activate oxidative stress genes and multiple antibiotic resistance in Escherichia coli. *Journal of Bacteriology*, *176*(1), 143–148.

Armelao, L., Barreca, D., Bottaro, G., Gasparotto, A., Maccato, C., Cinzia Maragno, … Mahne, D. (2007). Photocatalytic and antibacterial activity of TiO 2 and Au/TiO 2 nanosystems. *Nanotechnology*, *18*(37), 375709. https://doi.org/10.1088/0957-4484/18/37/375709

Asahi, R., Morikawa, T., Ohwaki, T., Aoki, K., & Taga, Y. (2001). Visible-Light Photocatalysis in Nitrogen-Doped Titanium Oxides. *Science*, *293*(5528), 269–271. https://doi.org/10.1126/science.1061051

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., … Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*, *2*, 2006.0008. https://doi.org/10.1038/msb4100050

Balagué, C., & Véscovi, E. G. (2001). Activation of Multiple Antibiotic Resistance in Uropathogenic Escherichia coli Strains by Aryloxoalcanoic Acid Compounds. *Antimicrobial Agents and Chemotherapy*, *45*(6), 1815–1822. https://doi.org/10.1128/AAC.45.6.1815-1822.2001

Baucheron, S., Tyler, S., Boyd, D., Mulvey, M. R., Chaslus-Dancla, E., & Cloeckaert, A. (2004). AcrAB-TolC Directs Efflux-Mediated Multidrug Resistance in Salmonella enterica Serovar Typhimurium DT104. *Antimicrobial Agents and Chemotherapy*, *48*(10), 3729–3735. https://doi.org/10.1128/AAC.48.10.3729-3735.2004

Ben-Israel, O., Ben-Israel, H., & Ulitzur, S. (1998). Identification and Quantification of Toxic Chemicals by Use of Escherichia coli Carryinglux Genes Fused to Stress Promoters. *Applied and Environmental Microbiology*, *64*(11), 4346–4352.

Benndorf, D., Thiersch, M., Loffhagen, N., Kunath, C., & Harms, H. (2006). Pseudomonas putida KT2440 responds specifically to chlorophenoxy herbicides and their initial metabolites. *PROTEOMICS*, *6*(11), 3319–3329. https://doi.org/10.1002/pmic.200500781

Bérdy, J. (2012). Thoughts and facts about antibiotics: Where we are now and where we are heading. *The Journal of Antibiotics*, *65*(8), 441–441. https://doi.org/10.1038/ja.2012.54

Bhalla, A., Pultz, N. J., Gries, D. M., Ray, A. J., Eckstein, E. C., Aron, D. C., & Donskey, C. J. (2004). Acquisition of nosocomial pathogens on hands after contact with environmental surfaces near hospitalized patients. *Infection Control & Hospital Epidemiology*, *25*(2), 164–167.

Bindels, D. S., Haarbosch, L., van Weeren, L., Postma, M., Wiese, K. E., Mastop, M., … Gadella Jr, T. W. J. (2017). mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nature Methods*, *14*(1), 53–56. https://doi.org/10.1038/nmeth.4074

Bintsis Thomas, Litopoulou-Tzanetaki Evanthia, & Robinson Richard K. (2000). Existing and potential applications of ultraviolet light in the food industry – a critical review. *Journal of the Science of Food and Agriculture*, *80*(6), 637–645. https://doi.org/10.1002/(SICI)1097-0010(20000501)80:6<637::AID-JSFA603>3.0.CO;2-1

Blair, J. M., & Piddock, L. J. (2009). Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. *Current Opinion in Microbiology*, *12*(5), 512–519. https://doi.org/10.1016/j.mib.2009.07.003

Block, S. S., Seng, V. P., & Goswami, D. W. (1997). Chemically Enhanced Sunlight for Killing Bacteria. *Journal of Solar Energy Engineering*, *119*(1), 85–91. https://doi.org/10.1115/1.2871858

Breidenstein, E. B. M., de la Fuente-Núñez, C., & Hancock, R. E. W. (2011). Pseudomonas aeruginosa: all roads lead to resistance. *Trends in Microbiology*, *19*(8), 419–426. https://doi.org/10.1016/j.tim.2011.04.005

Brook, L. A., Evans, P., Foster, H. A., Pemble, M. E., Steele, A., Sheel, D. W., & Yates, H. M. (2007). Highly bioactive silver and silver/titania composite films grown by chemical vapour deposition. *Journal of Photochemistry and Photobiology A: Chemistry*, *187*(1), 53–63. https://doi.org/10.1016/j.jphotochem.2006.09.014

Caballero, L., Whitehead, K. A., Allen, N. S., & Verran, J. (2009). Inactivation of Escherichia coli on immobilized TiO2 using fluorescent light. *Journal of Photochemistry and Photobiology A: Chemistry*, *202*(2–3), 92–98. https://doi.org/10.1016/j.jphotochem.2008.11.005

Campoccia, D., Montanaro, L., & Arciola, C. R. (2013). A review of the biomaterials technologies for infection-resistant surfaces. *Biomaterials*, *34*(34), 8533–8554. https://doi.org/10.1016/j.biomaterials.2013.07.089

Casey, A. L., Adams, D., Karpanen, T. J., Lambert, P. A., Cookson, B. D., Nightingale, P., … Elliott, T. S. J. (2010). Role of copper in reducing hospital environment contamination. *Journal of Hospital Infection*, *74*(1), 72–77. https://doi.org/10.1016/j.jhin.2009.08.018

Chacko, B., Thomas, K., David, T., Paul, H., Jeyaseelan, L., & Peter, J. V. (2017). Attributable cost of a nosocomial infection in the intensive care unit: A prospective cohort study. *World Journal of Critical Care Medicine*, *6*(1), 79–84. https://doi.org/10.5492/wjccm.v6.i1.79

Charles, P. G., & Grayson, M. L. (2004). The dearth of new antibiotic development: why we should be worried and what we can do about it. *Medical Journal of Australia*, *181*, 549–553.

Chen, X., Zheng, Z., Ke, X., Jaatinen, E., Xie, T., Wang, D., … Zhu, H. (2010). Supported silver nanoparticles as photocatalysts under ultraviolet and visible light irradiation. *Green Chemistry*, *12*(3), 414–419. https://doi.org/10.1039/B921696K

Choi, W., Termin, A., & Hoffmann, M. R. (1994). The role of metal ion dopants in quantum-sized TiO2: correlation between photoreactivity and charge carrier recombination dynamics. *The Journal of Physical Chemistry*, *98*(51), 13669–13679.

Climo, M. W., Yokoe, D. S., Warren, D. K., Perl, T. M., Bolon, M., Herwaldt, L. A., … Wong, E. S. (2013). Effect of Daily Chlorhexidine Bathing on Hospital-Acquired Infection. *New England Journal of Medicine*, *368*(6), 533–542. https://doi.org/10.1056/NEJMoa1113849

Cohen, S. P., Levy, S. B., Foulds, J., & Rosner, J. L. (1993). Salicylate induction of antibiotic resistance in Escherichia coli: activation of the mar operon and a mar-independent pathway. *Journal of Bacteriology*, *175*(24), 7856–7862. https://doi.org/10.1128/jb.175.24.7856-7862.1993

Commodity and Metal Prices, Metal Price Charts - InvestmentMine. (n.d.). Retrieved April 17, 2018, from http://www.infomine.com/investment/metal-prices/

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology*, *303*(6), 287–292. https://doi.org/10.1016/j.ijmm.2013.02.009

Dalrymple, O. K., Stefanakos, E., Trotz, M. A., & Goswami, D. Y. (2010). A review of the mechanisms and modeling of photocatalytic disinfection. *Applied Catalysis B: Environmental*, *98*(1–2), 27–38. https://doi.org/10.1016/j.apcatb.2010.05.001

Dancer, S. J. (2011). Hospital cleaning in the 21st century. *European Journal of Clinical Microbiology & Infectious Diseases*, *30*(12), 1473–1481. https://doi.org/10.1007/s10096-011-1250-x

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science*, *264*(5157), 375–382. https://doi.org/10.1126/science.8153624

Delihas, N., & Forst, S. (2001). MicF: an antisense RNA gene involved in response of Escherichia coli to global stress factors. *Journal of Molecular Biology*, *313*(1), 1–12. https://doi.org/10.1006/jmbi.2001.5029

Dollwet, H., & Sorenson, J. (1985). Historic uses of copper compounds in medicine. *Trace Elements in Medicine*, *2*(2), 80–87.

Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J., & Chopra, I. (2008). Increased mutability of Pseudomonas aeruginosa in biofilms. *Journal of Antimicrobial Chemotherapy*, *61*(5), 1053–1056. https://doi.org/10.1093/jac/dkn044

Du, D., Wang, Z., James, N. R., Voss, J. E., Klimont, E., Ohene-Agyei, T., … Luisi, B. F. (2014). Structure of the AcrAB-TolC multidrug efflux pump. *Nature*, *509*(7501), 512–515. https://doi.org/10.1038/nature13205

Dupont, M., James, C. E., Chevalier, J., & Pagès, J.-M. (2007). An Early Response to Environmental Stress Involves Regulation of OmpX and OmpF, Two Enterobacterial Outer Membrane Pore-Forming Proteins. *Antimicrobial Agents and Chemotherapy*, *51*(9), 3190–3198. https://doi.org/10.1128/AAC.01481-06

Fenoglio Ivana, Greco Giovanna, Livraghi Stefano, & Fubini Bice. (2009). Non-UV-Induced Radical Reactions at the Surface of TiO2 Nanoparticles That May Trigger Toxic Responses. *Chemistry – A European Journal*, *15*(18), 4614–4621. https://doi.org/10.1002/chem.200802542

Fernández, L., Breidenstein, E. B. M., & Hancock, R. E. W. (2011). Creeping baselines and adaptive resistance to antibiotics. *Drug Resistance Updates*, *14*(1), 1–21. https://doi.org/10.1016/j.drup.2011.01.001

Fernández, L., & Hancock, R. E. W. (2012). Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance. *Clinical Microbiology Reviews*, *25*(4), 661–681. https://doi.org/10.1128/CMR.00043-12

Foster, H. A., Ditta, I. B., Varghese, S., & Steele, A. (2011). Photocatalytic disinfection using titanium dioxide: spectrum and mechanism of antimicrobial activity. *Applied Microbiology and Biotechnology*, *90*(6), 1847–1868. https://doi.org/10.1007/s00253-011-3213-7

Franke, S., Grass, G., Rensing, C., & Nies, D. H. (2003). Molecular Analysis of the Copper-Transporting Efflux System CusCFBA of Escherichia coli. *Journal of Bacteriology*, *185*(13), 3804–3812. https://doi.org/10.1128/JB.185.13.3804-3812.2003

Fujishima, A., Rao, T. N., & Tryk, D. A. (2000). Titanium dioxide photocatalysis. *Journal of Photochemistry and Photobiology C: Photochemistry Reviews*, *1*(1), 1–21. https://doi.org/10.1016/S1389-5567(00)00002-2

Fujishima, A., & Zhang, X. (2006). Titanium dioxide photocatalysis: present situation and future approaches. *Comptes Rendus Chimie*, *9*(5–6), 750–760.

Gefen, O., Fridman, O., Ronin, I., & Balaban, N. Q. (2014). Direct observation of single stationary-phase bacteria reveals a surprisingly long period of constant protein production activity. *Proceedings of the National Academy of Sciences*, *111*(1), 556–561. https://doi.org/10.1073/pnas.1314114111

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison Iii, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343–345. https://doi.org/10.1038/nmeth.1318

Gibson, P. S. (2016). The biochemistry and genetics of herbicide-induced changes in antibiotic resistance in Salmonella enterica and Escherichia coli. Retrieved from https://ir.canterbury.ac.nz/handle/10092/12033

Głuzek, J., Masalski, J., Furman, P., & Nitsch, K. (1997). Structural and electrochemical examinations of PACVD TiO2 films in Ringer solution. *Biomaterials*, *18*(11), 789–794. https://doi.org/10.1016/S0142-9612(96)00210-4

Goodman, E. R., Piatt, R., Bass, R., Onderdonk, A. B., Yokoe, D. S., & Huang, S. S. (2008). Impact of an Environmental Cleaning Intervention on the Presence of Methicillin-Resistant Staphylococcus aureus and Vancomycin-Resistant Enterococci on Surfaces in Intensive Care Unit Rooms. *Infection Control & Hospital Epidemiology*, *29*(7), 593–599. https://doi.org/10.1086/588566

Gordon, A. S., Howell, L. D., & Harwood, V. (1994). Responses of diverse heterotrophic bacteria to elevated copper concentrations. *Canadian Journal of Microbiology*, *40*(5), 408–411.

Grass, G., Rensing, C., & Solioz, M. (2011). Metallic Copper as an Antimicrobial Surface. *Applied and Environmental Microbiology*, *77*(5), 1541–1547. https://doi.org/10.1128/AEM.02766-10

Greenberg, J. T., Monach, P., Chou, J. H., Josephy, P. D., & Demple, B. (1990). Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in Escherichia coli. *Proceedings of the National Academy of Sciences*, *87*(16), 6181–6185. https://doi.org/10.1073/pnas.87.16.6181

Grenier, F., Matteau, D., Baby, V., & Rodrigue, S. (2014). Complete Genome Sequence of Escherichia coli BW25113. *Genome Announcements*, *2*(5). https://doi.org/10.1128/genomeA.01038-14

Griffith, C. J., Cooper, R. A., Gilmore, J., Davies, C., & Lewis, M. (2000). An evaluation of hospital cleaning regimes and standards. *Journal of Hospital Infection*, *45*(1), 19–28. https://doi.org/10.1053/jhin.1999.0717

Gu, M., & Imlay, J. A. (2011). The SoxRS response of Escherichia coli is directly activated by redox-cycling drugs rather than by superoxide. *Molecular Microbiology*, *79*(5), 1136–1150. https://doi.org/10.1111/j.1365-2958.2010.07520.x

Guo, X., Hao, C., Jin, G., Zhu, H.-Y., & Guo, X.-Y. (2014). Copper Nanoparticles on Graphene Support: An Efficient Photocatalyst for Coupling of Nitroaromatics in Visible Light. *Angewandte Chemie International Edition*, *53*(7), 1973–1977. https://doi.org/10.1002/anie.201309482

Hao, Z., Lou, H., Zhu, R., Zhu, J., Zhang, D., Zhao, B. S., … Chen, P. R. (2014). The multiple antibiotic resistance regulator MarR is a copper sensor in Escherichia coli. *Nature Chemical Biology; Cambridge*, *10*(1), 21–28. http://dx.doi.org.ezproxy.canterbury.ac.nz/10.1038/nchembio.1380

Hashimoto, Kazuhito, Irie, H., & Fujishima, A. (2005). TiO2 Photocatalysis: A Historical Overview and Future Prospects. *Japanese Journal of Applied Physics*, *44*(12R), 8269. https://doi.org/10.1143/JJAP.44.8269

Hashimoto, Ko, Kokubun, S., Itoi, E., & Roach, H. I. (2007). Improved quantification of DNA methylation using methylation-sensitive restriction enzymes and real-time PCR. *Epigenetics*, *2*(2), 86–91.

Hassan, M., Tuckman, H. P., Patrick, R. H., Kountz, D. S., & Kohn, J. L. (2010). Cost of hospital-acquired infection. *Hospital Topics*, *88*(3), 82–89.

Hassing, R.-J., Goessens, W. H. F., Mevius, D. J., Pelt, W. van, Mouton, J. W., Verbon, A., & Genderen, P. J. van. (2013). Decreased ciprofloxacin susceptibility in <Emphasis Type="Italic">Salmonella</Emphasis> Typhi and Paratyphi infections in ill-returned travellers: the impact on clinical outcome and future treatment options. *European Journal of*

Clinical Microbiology & Infectious Diseases, *32*(10), 1295–1301. https://doi.org/10.1007/s10096-013-1878-9

Hausner, M., & Wuertz, S. (1999). High Rates of Conjugation in Bacterial Biofilms as Determined by Quantitative In Situ Analysis. *Applied and Environmental Microbiology*, *65*(8), 3710–3713.

Heinemann, J. A., Ankenbauer, R. G., & Amábile-Cuevas, C. F. (2000). Do antibiotics maintain antibiotic resistance? *Drug Discovery Today*, *5*(5), 195–204.

Hoek, V., H.a.m, A., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., & Aarts, H. J. M. (2011). Acquired Antibiotic Resistance Genes: An Overview. *Frontiers in Microbiology*, *2*. https://doi.org/10.3389/fmicb.2011.00203

ISO, 27447. (2009). ISO 27447:2009 - Fine ceramics (advanced ceramics, advanced technical ceramics) -- Test method for antibacterial activity of semiconducting photocatalytic materials. Retrieved March 19, 2017, from https://www.iso.org/standard/44156.html

Janoir, C., Zeller, V., Kitzis, M. D., Moreau, N. J., & Gutmann, L. (1996). High-level fluoroquinolone resistance in Streptococcus pneumoniae requires mutations in parC and gyrA. *Antimicrobial Agents and Chemotherapy*, *40*(12), 2760–2764.

Jiang, W., Wang, X., Wu, Z., Yue, X., Yuan, S., Lu, H., & Liang, B. (2015). Silver Oxide as Superb and Stable Photocatalyst under Visible and Near-Infrared Light Irradiation and Its Photocatalytic Mechanism. *Industrial & Engineering Chemistry Research*, *54*(3), 832–841. https://doi.org/10.1021/ie503241k

Jun, H. (2017). The effects of sub-lethal concentrations of biocides copper, pyrethrins and atrazine on antibiotic tolerance of Escherichia coli. Retrieved from https://ir.canterbury.ac.nz/handle/10092/13419

Jung, W. K., Koo, H. C., Kim, K. W., Shin, S., Kim, S. H., & Park, Y. H. (2008). Antibacterial Activity and Mechanism of Action of the Silver Ion in Staphylococcus aureus and Escherichia coli. *Applied and Environmental Microbiology*, *74*(7), 2171–2178. https://doi.org/10.1128/AEM.02001-07

Kannan, S., Sams, T., Maury, J., & Workman, C. T. (2018). Reconstructing Dynamic Promoter Activity Profiles from Reporter Gene Data. *ACS Synthetic Biology*.

Karpanen, T. J., Casey, A. L., Lambert, P. A., Cookson, B. D., Nightingale, P., Miruszenko, L., & Elliott, T. S. J. (2012). The Antimicrobial Efficacy of Copper Alloy Furnishing in the Clinical Environment: A Crossover Study. *Infection Control and Hospital Epidemiology*, *33*(1), 3–9. https://doi.org/10.1086/663644

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)*, *28*(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Khan, S. R., Gaines, J., Roop, R. M., & Farrand, S. K. (2008). Broad-Host-Range Expression Vectors with Tightly Regulated Promoters and Their Use To Examine the Influence of TraR and TraM Expression on Ti Plasmid Quorum Sensing. *Applied and Environmental Microbiology*, *74*(16), 5053–5062. https://doi.org/10.1128/AEM.01098-08

Kjellander, J. O., Klein, J. O., & Finland, M. (1963). In vitro Activity of Penicillins Against Staphylococcus albus. *Proceedings of the Society for Experimental Biology and Medicine*, *113*(4), 1023–1031. https://doi.org/10.3181/00379727-113-28563

Klasen, H. J. (2000). Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns*, *26*(2), 117–130. https://doi.org/10.1016/S0305-4179(99)00108-4

Klevens, R. M., Edwards, J. R., Richards Jr, C. L., Horan, T. C., Gaynes, R. P., Pollock, D. A., & Cardo, D. M. (2007). Estimating health care-associated infections and deaths in US hospitals, 2002. *Public Health Reports*, *122*(2), 160–166.

Klevens, R. M., Edwards, J. R., Tenover, F. C., McDonald, L. C., Horan, T., & Gaynes, R. (2006). Changes in the Epidemiology of Methicillin-Resistant Staphylococcus aureus in Intensive Care Units in US Hospitals, 1992–2003. *Clinical Infectious Diseases*, *42*(3), 389–391. https://doi.org/10.1086/499367

Klotsky, R.-A., & Schwartz, I. (1987). Measurement of cat expression from growth-rate-regulated promoters employing β-lactamase activity as an indicator of plasmid copy number. *Gene*, *55*(1), 141–146. https://doi.org/10.1016/0378-1119(87)90257-5

Kobayashi, K., Tsukagoshi, N., & Aono, R. (2001). Suppression of hypersensitivity of Escherichia coli acrB mutant to organic solvents by integrational activation of the acrEF operon with the IS1 or IS2 element. *Journal of Bacteriology*, *183*(8), 2646–2653. https://doi.org/10.1128/JB.183.8.2646-2653.2001

Konstantinou, I. K., & Albanis, T. A. (2004). TiO2-assisted photocatalytic degradation of azo dyes in aqueous solution: kinetic and mechanistic investigations: A review. *Applied Catalysis B: Environmental*, *49*(1), 1–14. https://doi.org/10.1016/j.apcatb.2003.11.010

Kovach, M. E., Elzer, P. H., Steven Hill, D., Robertson, G. T., Farris, M. A., Roop, R. M., & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, *166*(1), 175–176. https://doi.org/10.1016/0378-1119(95)00584-1

Kramer, A., Schwebke, I., & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infectious Diseases*, *6*, 130. https://doi.org/10.1186/1471-2334-6-130

Kubacka, A., Muñoz-Batista, M. J., Ferrer, M., & Fernández-García, M. (2013). UV and visible light optimization of anatase TiO2 antimicrobial properties: Surface deposition of metal and oxide (Cu, Zn, Ag) species. *Applied Catalysis B: Environmental*, *140–141*, 680–690. https://doi.org/10.1016/j.apcatb.2013.04.077

Kuhn, P. (1983). Doorknobs: A Source of Nosocomial Infection? *Diagnostic Medicine*.

Kunin, C. M. (1993). Resistance to Antimicrobial Drugs—A Worldwide Calamity. *Annals of Internal Medicine*, *118*(7), 557. https://doi.org/10.7326/0003-4819-118-7-199304010-00011

Kurenbach, B., Gibson, P. S., Hill, A. M., Bitzer, A. S., Silby, M. W., Godsoe, W., & Heinemann, J. A. (2017). Herbicide ingredients change Salmonella enterica sv. Typhimurium and Escherichia coli antibiotic responses. *Microbiology*, *163*(12), 1791–1801. https://doi.org/10.1099/mic.0.000573

Kurenbach, B., Marjoshi, D., Amábile-Cuevas, C. F., Ferguson, G. C., Godsoe, W., Gibson, P., & Heinemann, J. A. (2015a). Sublethal Exposure to Commercial Formulations of the Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate Cause Changes in Antibiotic Susceptibility in Escherichia coli and Salmonella enterica serovar Typhimurium. *MBio*, *6*(2), e00009-15. https://doi.org/10.1128/mBio.00009-15

Kurenbach, B., Marjoshi, D., Amábile-Cuevas, C. F., Ferguson, G. C., Godsoe, W., Gibson, P., & Heinemann, J. A. (2015b). Sublethal Exposure to Commercial Formulations of the Herbicides Dicamba, 2,4-Dichlorophenoxyacetic Acid, and Glyphosate Cause Changes in Antibiotic Susceptibility in Escherichia coli and Salmonella enterica serovar Typhimurium. *MBio*, *6*(2), e00009-15. https://doi.org/10.1128/mBio.00009-15

Kwon, H. J., Bennik, M. H. J., Demple, B., & Ellenberger, T. (2000). Crystal structure of the *Escherichia coli* Rob transcription factor in complex with DNA. *Nature Structural & Molecular Biology*, *7*(5), 424–430. https://doi.org/10.1038/75213

Lee, C., Kim, J., Shin, S. G., & Hwang, S. (2006). Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. *Journal of Biotechnology*, *123*(3), 273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014

Lee, D., Krumdieck, S., & Talwar, S. D. (2013). Scale-up design for industrial development of a PP-MOCVD coating system. *Surface and Coatings Technology*, *230*, 39–45.

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nature Reviews Microbiology*, *4*(7), 556.

Li, Q., Xie, R., Li, Y. W., Mintz, E. A., & Shang, J. K. (2007). Enhanced Visible-Light-Induced Photocatalytic Disinfection of E. coli by Carbon-Sensitized Nitrogen-Doped Titanium Oxide. *Environmental Science & Technology*, *41*(14), 5050–5056. https://doi.org/10.1021/es062753c

Li, X.-Z., & Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. *Drugs*, *64*(2), 159–204.

Li, Z., & Demple, B. (1994). SoxS, an activator of superoxide stress genes in Escherichia coli. Purification and interaction with DNA. *Journal of Biological Chemistry*, *269*(28), 18371–18377.

Lin, H. T., Bavro, V. N., Barrera, N. P., Frankish, H. M., Velamakanni, S., van Veen, H. W., … Walmsley, A. R. (2009). MacB ABC transporter is a dimer whose ATPase activity and macrolide-binding capacity are regulated by the membrane fusion protein MacA. *The Journal of Biological Chemistry*, *284*(2), 1145–1154. https://doi.org/10.1074/jbc.M806964200

Lin, J., Nishino, K., Roberts, M. C., Tolmasky, M., Aminov, R. I., & Zhang, L. (2015). Mechanisms of antibiotic resistance. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00034

Liu Jincheng, Bai Hongwei, Wang Yinjie, Liu Zhaoyang, Zhang Xiwang, & Sun Darren Delai. (2010). Self-Assembling TiO2 Nanorods on Large Graphene Oxide Sheets at a Two-Phase Interface and Their Anti-Recombination in Photocatalytic Applications. *Advanced Functional Materials*, *20*(23), 4175–4181. https://doi.org/10.1002/adfm.201001391

Lopilato, J., Bortner, S., & Beckwith, J. (1986). Mutations in a new chromosomal gene of <Emphasis Type="Italic">Escherichia coli</Emphasis> K-12, <Emphasis Type="Italic">pcnB</Emphasis>, reduce plasmid copy number of pBR322 and its derivatives. *Molecular and General Genetics MGG*, *205*(2), 285–290. https://doi.org/10.1007/BF00430440

Majiduddin, F. K., Materon, I. C., & Palzkill, T. G. (2002). Molecular analysis of beta-lactamase structure and function. *International Journal of Medical Microbiology*, *292*(2), 127–137.

Martin, R. G., & Rosner, J. L. (1995). Binding of purified multiple antibiotic-resistance repressor protein (MarR) to mar operator sequences. *Proceedings of the National Academy of Sciences*, *92*(12), 5456–5460.

Mathews, N. R., Morales, E. R., Cortés-Jacome, M. A., & Toledo Antonio, J. A. (2009). TiO2 thin films – Influence of annealing temperature on structural, optical and photocatalytic properties. *Solar Energy*, *83*(9), 1499–1508. https://doi.org/10.1016/j.solener.2009.04.008

Mehtar, S., Wiid, I., & Todorov, S. D. (2008). The antimicrobial activity of copper and copper alloys against nosocomial pathogens and Mycobacterium tuberculosis isolated from healthcare facilities in the Western Cape: an in-vitro study. *Journal of Hospital Infection*, *68*(1), 45–51. https://doi.org/10.1016/j.jhin.2007.10.009

Michaelidis, C. I., Fine, M. J., Lin, C. J., Linder, J. A., Nowalk, M. P., Shields, R. K., … Smith, K. J. (2016). The hidden societal cost of antibiotic resistance per antibiotic prescribed in the

United States: an exploratory analysis. *BMC Infectious Diseases*, *16*, 655. https://doi.org/10.1186/s12879-016-1990-4

Mills, A., Davies, R. H., & Worsley, D. (1993). Water purification by semiconductor photocatalysis. *Chemical Society Reviews*, *22*(6), 417–425. https://doi.org/10.1039/CS9932200417

Mills, A., Hill, C., & Robertson, P. K. J. (2012). Overview of the current ISO tests for photocatalytic materials. *Journal of Photochemistry and Photobiology A: Chemistry*, *237*, 7–23. https://doi.org/10.1016/j.jphotochem.2012.02.024

Ministry of Health NZ. (2012). Health Expenditure Trends in New Zealand 2000–2010. Retrieved April 5, 2018, from https://www.health.govt.nz/publication/health-expenditure-trends-new-zealand-2000-2010

Molin, S., & Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology*, *14*(3), 255–261. https://doi.org/10.1016/S0958-1669(03)00036-3

Morona, R., Manning, P. A., & Reeves, P. (1983). Identification and characterization of the TolC protein, an outer membrane protein from Escherichia coli. *Journal of Bacteriology*, *153*(2), 693–699.

Motta, S. S., Cluzel, P., & Aldana, M. (2015). Adaptive Resistance in Bacteria Requires Epigenetic Inheritance, Genetic Noise, and Cost of Efflux Pumps. *PLOS ONE*, *10*(3), e0118464. https://doi.org/10.1371/journal.pone.0118464

Motulsky, H. (1999). *Analyzing data with GraphPad prism*. GraphPad Software Incorporated.

Muranyi, P., Schraml, C., & Wunderlich, J. (2010). Antimicrobial efficiency of titanium dioxide-coated surfaces. *Journal of Applied Microbiology*, *108*(6), 1966–1973. https://doi.org/10.1111/j.1365-2672.2009.04594.x

Nahim-Granados, S., Sánchez Pérez, J. A., & Polo-Lopez, M. I. (2017). Effective solar processes in fresh-cut wastewater disinfection: Inactivation of pathogenic E. coli O157:H7 and Salmonella enteritidis. *Catalysis Today*. https://doi.org/10.1016/j.cattod.2017.10.042

Nishino, K., Nikaido, E., & Yamaguchi, A. (2007). Regulation of Multidrug Efflux Systems Involved in Multidrug and Metal Resistance of Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology*, *189*(24), 9066–9075. https://doi.org/10.1128/JB.01045-07

Nunoshiba, T., Hidalgo, E., Cuevas, C. F. A., & Demple, B. (1992). Two-stage control of an oxidative stress regulon: the Escherichia coli SoxR protein triggers redox-inducible expression of the soxS regulatory gene. *Journal of Bacteriology*, *174*(19), 6054–6060. https://doi.org/10.1128/jb.174.19.6054-6060.1992

Olaitan, A. O., Morand, S., & Rolain, J.-M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00643

Pal, A., Pehkonen, S. O., Yu, L. E., & Ray, M. B. (2007). Photocatalytic inactivation of Gram-positive and Gram-negative bacteria using fluorescent light. *Journal of Photochemistry and Photobiology A: Chemistry*, *186*(2–3), 335–341. https://doi.org/10.1016/j.jphotochem.2006.09.002

Pelaez, M., Nolan, N. T., Pillai, S. C., Seery, M. K., Falaras, P., Kontos, A. G., … Dionysiou, D. D. (2012). A review on the visible light active titanium dioxide photocatalysts for environmental applications. *Applied Catalysis B: Environmental*, *125*, 331–349. https://doi.org/10.1016/j.apcatb.2012.05.036

Piddock, L. J. (2015). Teixobactin, the first of a new class of antibiotics discovered by iChip technology? *Journal of Antimicrobial Chemotherapy*, *70*(10), 2679–2680.

Piddock, L. J. V. (2012). The crisis of no new antibiotics-what is the way forward? *The Lancet Infectious Diseases; London*, *12*(3), 249–253.

Prajapat, M. K., Jain, K., & Saini, S. (2015). Control of MarRAB Operon in Escherichia coli via Autoactivation and Autorepression. *Biophysical Journal*, *109*(7), 1497–1508. https://doi.org/10.1016/j.bpj.2015.08.017

Price, C. T. ., Lee, I. R., & Gustafson, J. E. (2000). The effects of salicylate on bacteria. *The International Journal of Biochemistry & Cell Biology*, *32*(10), 1029–1043. https://doi.org/10.1016/S1357-2725(00)00042-X

Rand, J. D., Danby, S. G., Greenway, D. L., & England, R. R. (2002). Increased expression of the multidrug efflux genes acrAB occurs during slow growth of Escherichia coli. *FEMS Microbiology Letters*, *207*(1), 91–95.

Remus-Emsermann, M. N. P., Gisler, P., & Drissner, D. (2016). MiniTn7-transposon delivery vectors for inducible or constitutive fluorescent protein expression in Enterobacteriaceae. *FEMS Microbiology Letters*, *363*(16). https://doi.org/10.1093/femsle/fnw178

Renwick, M. J., Brogan, D. M., & Mossialos, E. (2016). A systematic review and critical assessment of incentive strategies for discovery and development of novel antibiotics. *The Journal of Antibiotics*, *69*(2), 73–88. https://doi.org/10.1038/ja.2015.98

Rosenberg, E. Y., Bertenthal, D., Nilles, M. L., Bertrand, K. P., & Nikaido, H. (2003). Bile salts and fatty acids induce the expression of Escherichia coli AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Molecular Microbiology*, *48*(6), 1609–1619.

RStudio Team. (2015). *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, Inc. Retrieved from http://www.rstudio.com/

Rupp, M. E., Fitzgerald, T., Marion, N., Helget, V., Puumala, S., Anderson, J. R., & Fey, P. D. (2004). Effect of silver-coated urinary catheters: Efficacy, cost-effectiveness, and antimicrobial resistance. *American Journal of Infection Control*, *32*(8), 445–450. https://doi.org/10.1016/j.ajic.2004.05.002

Russell A.D. (2002). Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *Journal of Applied Microbiology*, *92*(s1), 121S-135S. https://doi.org/10.1046/j.1365-2672.92.5s1.12.x

Santo, C. E., Lam, E. W., Elowsky, C. G., Quaranta, D., Domaille, D. W., Chang, C. J., & Grass, G. (2011). Bacterial Killing by Dry Metallic Copper Surfaces. *Applied and Environmental Microbiology*, *77*(3), 794–802. https://doi.org/10.1128/AEM.01599-10

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Schmidt, M. G., Attaway, H. H., Sharpe, P. A., John, J., Sepkowitz, K. A., Morgan, A., … Cantey, J. R. (2012). Sustained reduction of microbial burden on common hospital surfaces through introduction of copper. *Journal of Clinical Microbiology*, *50*(7), 2217–2223.

Schnappinger, D., & Hillen, W. (1996). Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of Microbiology*, *165*(6), 359–369. https://doi.org/10.1007/s002030050339

Scotto, V., Cintio, R. D., & Marcenaro, G. (1985). The influence of marine aerobic microbial film on stainless steel corrosion behaviour. *Corrosion Science*, *25*(3), 185–194. https://doi.org/10.1016/0010-938X(85)90094-0

Sekiguchi, K., Sanada, A., & Sakamoto, K. (2003). Degradation of toluene with an ozone-decomposition catalyst in the presence of ozone, and the combined effect of TiO2 addition. *Catalysis Communications*, *4*(5), 247–252. https://doi.org/10.1016/S1566-7367(03)00047-5

Sezonov, G., Joseleau-Petit, D., & D'Ari, R. (2007). Escherichia coli Physiology in Luria-Bertani Broth. *Journal of Bacteriology*, *189*(23), 8746–8749. https://doi.org/10.1128/JB.01368-07

Storz, G., & Imlayt, J. A. (1999). Oxidative stress. *Current Opinion in Microbiology*, *2*(2), 188–194. https://doi.org/10.1016/S1369-5274(99)80033-2

Tao, L., Jackson, R. E., Rouvière, P. E., & Cheng, Q. (2005). Isolation of chromosomal mutations that affect carotenoid production in Escherichia coli: mutations alter copy number of ColE1-type plasmids. *FEMS Microbiology Letters*, *243*(1), 227–233.

Touzé, T., Eswaran, J., Bokma, E., Koronakis, E., Hughes, C., & Koronakis, V. (2004). Interactions underlying assembly of the Escherichia coli AcrAB–TolC multidrug efflux system. *Molecular Microbiology*, *53*(2), 697–706. https://doi.org/10.1111/j.1365-2958.2004.04158.x

Tsai Ting-Mi, Chang Hsin-Hou, Chang Kia-Chih, Liu Yu-Lin, & Tseng Chun-Chieh. (2010). A comparative study of the bactericidal effect of photocatalytic oxidation by TiO2 on antibiotic-resistant and antibiotic-sensitive bacteria. *Journal of Chemical Technology & Biotechnology*, *85*(12), 1642–1653. https://doi.org/10.1002/jctb.2476

Vincent, M., Hartemann, P., & Engels-Deutsch, M. (2016). Antimicrobial applications of copper. *International Journal of Hygiene and Environmental Health*, *219*(7), 585–591. https://doi.org/10.1016/j.ijheh.2016.06.003

Walsh, C. T., & Wencewicz, T. A. (2014). Prospects for new antibiotics: a molecule-centered perspective. *The Journal of Antibiotics*, *67*(1), 7–22. https://doi.org/10.1038/ja.2013.49

Wang, S., Phillippy, A. M., Deng, K., Rui, X., Li, Z., Tortorello, M. L., & Zhang, W. (2010). Transcriptomic Responses of Salmonella enterica Serovars Enteritidis and Typhimurium to

Chlorine-Based Oxidative Stress. *Applied and Environmental Microbiology*, *76*(15), 5013–5024. https://doi.org/10.1128/AEM.00823-10

Webber, M. A., & Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy*, *51*(1), 9–11. https://doi.org/10.1093/jac/dkg050

Weisblum, B., Graham, M. Y., Gryczan, T., & Dubnau, D. (1979). Plasmid copy number control: isolation and characterization of high-copy-number mutants of plasmid pE194. *Journal of Bacteriology*, *137*(1), 635–643.

Wong, M.-S., Chu, W.-C., Sun, D.-S., Huang, H.-S., Chen, J.-H., Tsai, P.-J., … Chang, H.-H. (2006). Visible-Light-Induced Bactericidal Activity of a Nitrogen-Doped Titanium Photocatalyst against Human Pathogens. *Applied and Environmental Microbiology*, *72*(9), 6111–6116. https://doi.org/10.1128/AEM.02580-05

Xu, M., Lin, S., Chen, X., & Peng, Y. (2006). Studies on characteristics of nanostructure of N-TiO2 thin films and photo-bactericidal action. *Journal of Zhejiang University. Science. B*, *7*(7), 586–590. https://doi.org/10.1631/jzus.2006.B0586

Yamamoto, K., & Ishihama, A. (2005). Transcriptional response of Escherichia coli to external copper. *Molecular Microbiology*, *56*(1), 215–227. https://doi.org/10.1111/j.1365-2958.2005.04532.x

Zakharov, S. D., Sharma, O., Zhalnina, M., Yamashita, E., & Cramer, W. A. (2012). Pathways of colicin import: utilization of BtuB, OmpF porin and the TolC drug-export protein. *Biochemical Society Transactions*, *40*(6), 1463–1468. https://doi.org/10.1042/BST20120211

Zhang, A., Rosner, J. L., & Martin, R. G. (2008). Transcriptional activation by MarA, SoxS and Rob of two tolC promoters using one binding site: a complex promoter configuration for

tolC in Escherichia coli. *Molecular Microbiology*, *69*(6), 1450–1455. https://doi.org/10.1111/j.1365-2958.2008.06371.x

Zhang, J., Zhou, P., Liu, J., & Yu, J. (2014). New understanding of the difference of photocatalytic activity among anatase, rutile and brookite TiO 2. *Physical Chemistry Chemical Physics*, *16*(38), 20382–20386. https://doi.org/10.1039/C4CP02201G

Zhao, J., Milanova, M., Warmoeskerken, M. M. C. G., & Dutschk, V. (2012). Surface modification of TiO2 nanoparticles with silane coupling agents. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *413*, 273–279. https://doi.org/10.1016/j.colsurfa.2011.11.033

Zheng, M., Doan, B., Schneider, T. D., & Storz, G. (1999). OxyR and SoxRS Regulation offur. *Journal of Bacteriology*, *181*(15), 4639–4643.

Appendices

Appendix A: Design and Construction of Reporter Strains

This portion of methods was to design and construct a set of reporter strains that contained plasmids that contained a fluorescent gene whose expression was controlled by the promoter region of one of each of the efflux associated genes; *tolC, marR* or *soxS.*

A1 Primers designed for promoter regions of interest

The program Geneious (V10.1.3) (Kearse et al., 2012) was used to identify the genes *tolC, marR* and *soxS* and observe the fully sequenced genome of *E. coli* BW25113 and its annotations (Grenier et al., 2014, p. 25). The 204 basepair sequence upstream of the *tolC* transcriptional start was chosen. The 211 basepair upstream region was selected for *marR*. The 300 basepair upstream region was selected for *soxS.*

The gene *mScarlet* was selected as the fluorescence bioreporter. It was amplified from the plasmid pTriEx-RhoA-wt_mScarlet-i_SGFP2 (Bindels et al., 2017).

The plasmid pFru97 was selected to use as a vector for the gene construct (Remus-Emsermann et al., 2016).

Gibson assembly primers were designed using SnapGene or SnapGene Viewer software (GSL Biotech, Chicago, IL) to achieve the designed 3 plasmid constructs Figures A.1, A.2, and A.3. Primers were ordered from (primer company). And can be seen in Table A.2.

Table A. 1: *E. coli* gene promoter regions amplified. Genome regions selected for amplification from *E. coli* BW25113. Regions were upstream of transcription start for each gene.

Gene Name & Sequence location	Sequence
tolC 204bp upstream	TGTTAATGTCCTGGCACTAATAGTGAATTAAATGTGAATTTCAGCGACGT

of transcription start	TTGACTGCCGTTTGAGCAGTCATGTGTTAAATTGAGGCACATTAACGCCC TATGGCACGTAACGCCAACCTTTTGCGGTAGCGGCTTCTGCTAGAATCCG CAATAATTTTACAGTTTGATCGCGCTAAATACTGCTTCACCACAAGGAAT GCAA
marR 211bp upstream of transcription start	TGGTGGTTGTTATCCTGTGTATCTGGGTTATCAGCGAAAAGTATAAGGGG TAAACAAGGATAAAGTGTCACTCTTTAGCTAGCCTTGCATCGCATTGAAC AAAACTTGAACCGATTTAGCAAAACGTGGCATCGGTCAATTCATTCATTT GACTTATACTTGCCTGGGCAATATTATCCCCTGCAACTAATTACTTGCCA GGGCAACTAAT
soxS 300bp upstream of transcription start	AAATCTGCCTCTTTTCAGTGTTCAGTTCGTTAATTCATCTGTTGGGGAGT ATAATTCCTCAAGTTAACTTGAGGTAAAGCGATTTATGGAAAAGAAATTA CCCCGCATTAAAGCGCTGCTAACCCCCGGCGAAGTGGCGAAACGCAGCGG TGTGGCGGTATCGGCGCTGCATTTCTATGAAAGTAAAGGGTTGATTACCA GTATCCGTAACAGCGGCAATCAGCGGCGATATAAACGTGATGTGTTGCGA TATGTTGCAATTATCAAAATTGCTCAGCGTATTGGCATTCCGCTGGCGAC

Table A. 2: Primers used in this study. Blue regions bonded with vector. Red regions were overlapping primers. Black regions bonded with isolated gene regions on promoter and *mScarlet*.

Name	Target	Sequence	T_m (C_0)
Gisbon Assembly *Primers*			
FWD_primer_TolC	*tolC*	5' CAG GAC GCC CGC CAT AAA CTG CCA GGA ATT GGG GAT CGG ATG TTA ATG TCC TGG CAC TAA TAG TGA ATT AAA TGT 3’	87.1
REV_primer_TolC	*tolC*	5' TCG CCC TTG CTC ACC ATG GTT TGC ATT CCT TGT GGT GAA GCA G 3'	81.8
FWD_primer_SoxS	*soxS*	5' CAG GAC GCC CGC CAT AAA CTG CCA	89.9

		GGA ATT GGG GAT CGG AGT CGC CAG CGG AAT GC 3’	
REV_primer_SoxS	*soxS*	5' CCT CGC CCT TGC TCA CCA TGA AAT CTG CCT CTT TTC AGT GTT CAG TT 3'	81.5
FWD_primer_MarR	*marR*	5' CAG GAC GCC CGC CAT AAA CTG CCA GGA ATT GGG GAT CGG ATG GTG GTT GTT ATC CTG TGT ATC TGG 3'	88.9
REV_primer_MarR	*marR*	5' GCC TCG CCC TTG CTC ACC ATA TTA GTT GCC CTG GCA AGT AAT TAG T 3’	81.6
TolC_mScarlet_FWD	*tolC* and *mScarlet*	5' CTT CAC CAC AAG GAA TGC AAA CCA TGG TGA GCA AGG GC 3’	78.9
MarR_mScarlet_FWD	*marR* and *mScarlet*	5' TAC TTG CCA GGG CAA CTA ATA TGG TGA GCA AGG GCG AGG C 3’	81
SoxS_mScarlet_FWD	*soxS* and *mScarlet*	5' CAC TGA AAA GAG GCA GAT TTC ATG GTG AGC AAG GGC GAG GC 3’	80.9
mScarlet_REV	*mScarlet*	5' TTA CTG GAT CTA TCA ACA GGA GTC CAA GCT CAG CTA ATT ACT TGT ACA GCT CGT CCA TGC 3'	83.7

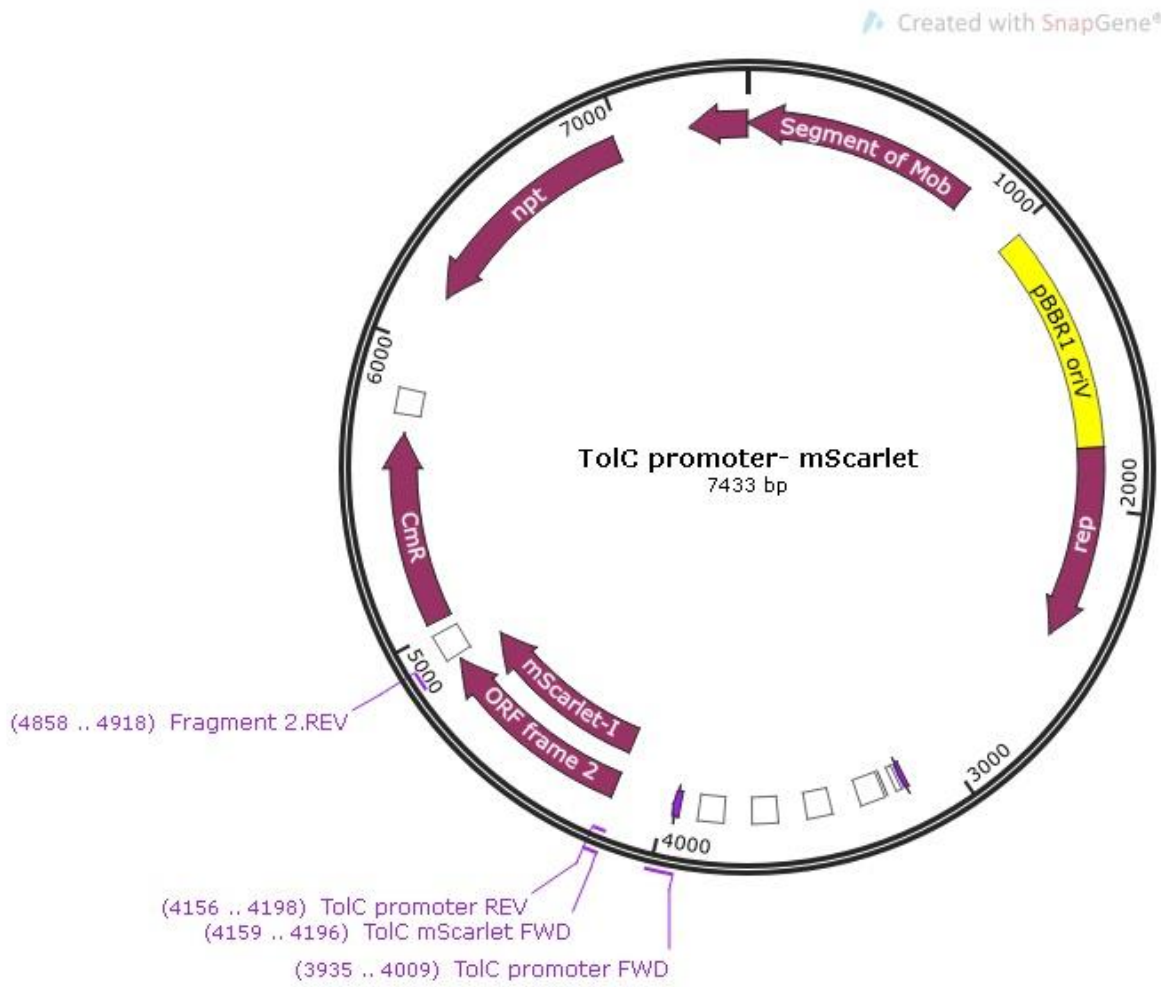


Figure A. 1: Plasmid map of pTolC-mScarlet. Relevant genes are marked out. Primer binding regions marked out. Promoter region marked out by between TolC Fwd and TolC Rev.

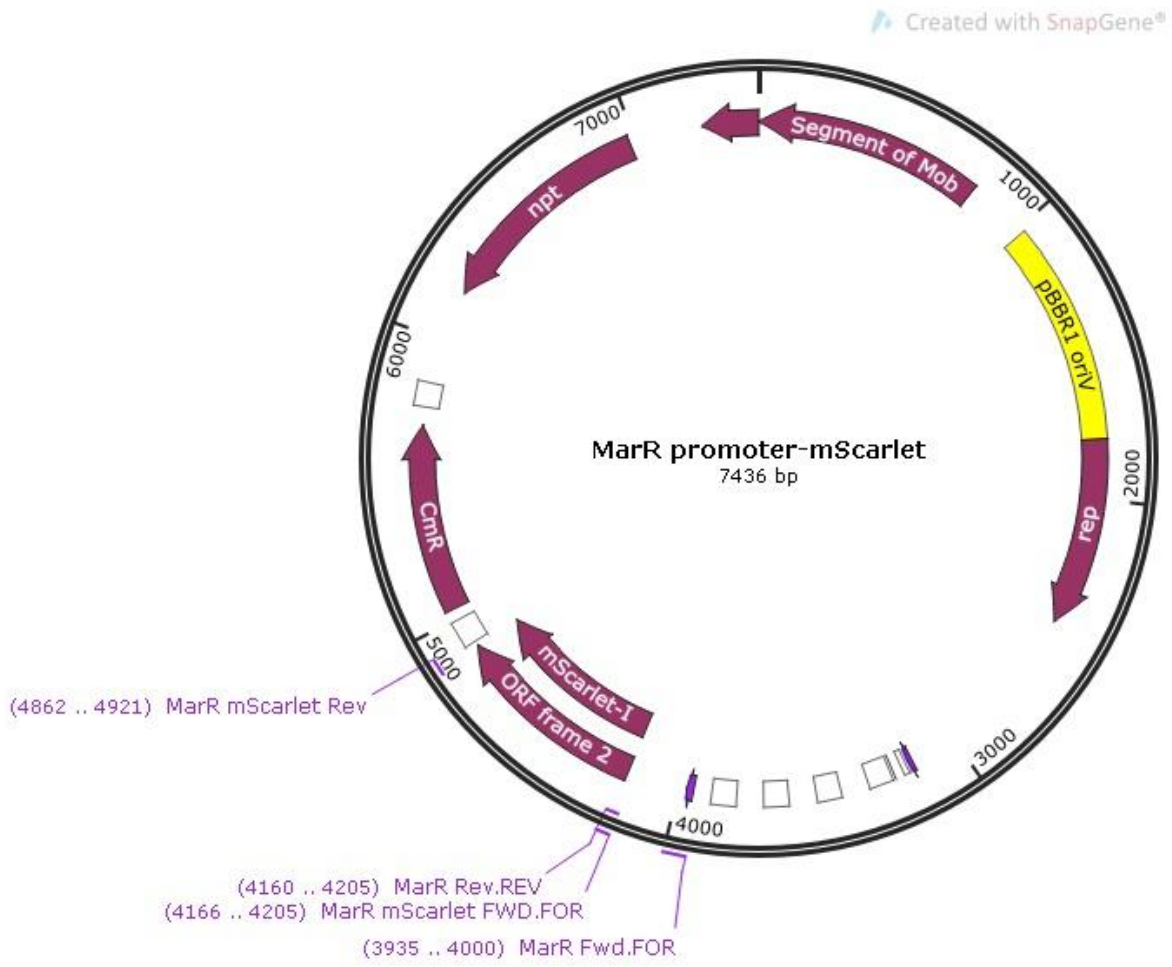


Figure A. 2: Plasmid map of pMarR-mScarlet. Relevant genes are marked out. Primer binding regions marked out. Promoter region marked out by between MarR Fwd and Mar Rev.

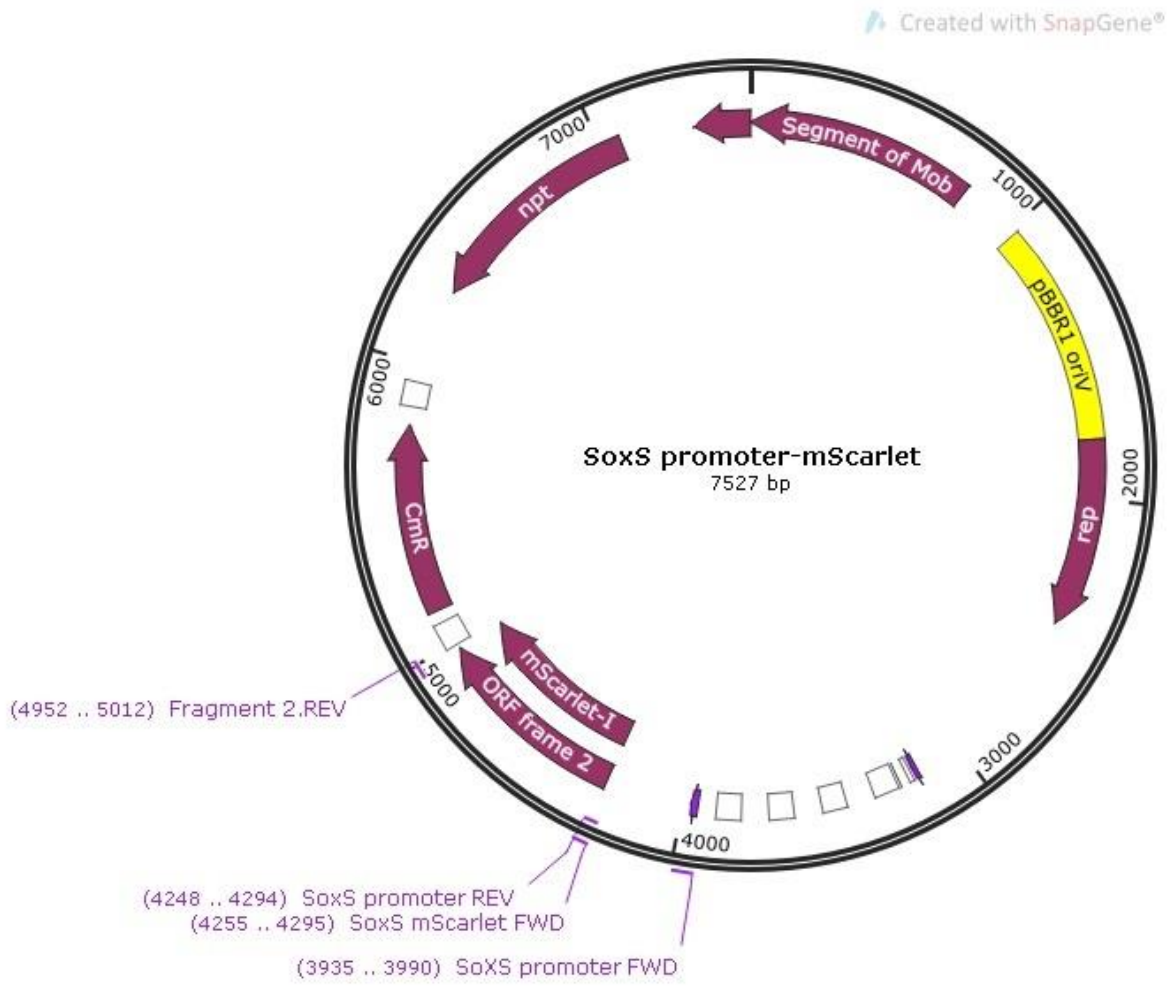


Figure A. 3: Plasmid map of pSoxS-mScarlet. Relevant genes are marked out. Primer binding regions marked out. Promoter region marked out by between SoxS Fwd and SoxS Rev.

A2 Amplification of Target Regions

DNA was extracted from *E. coli* BW25113. The promoter regions were amplified via PCR amplification according to the manufacturer's instructions (Roche; Mannheim, Germany) using Gibson assembly primers in Table A.2. *mScarlet-I* was amplified from the plasmid pTriEx-RhoA-wt_mScarlet-i_SGFP2.

A3 Gel Electrophoresis to isolate amplified DNA

1% agarose gels were made up using distilled water and HyAgarose^{TM} (HydraGene Co. Ltd) and heating to form a liquid gel. This was poured into gel mould and allowed to solidify. 3 µl

of SYBR® Safe DNA Gel Stain (Invitrogen™) was added to each DNA sample of 12 µl and then placed in gel wells. Charge was applied for 20min with a 100 bp-10,000 bp DNA ladder (SiZer™ -1000 plus) in wells on either side of the test wells.

The gels were examined for purified bands by observation under UV light. These bands for both *mScarlet* isolates and the promoters for *tolC, marR* and *soxS* can be seen in Figure A.4.

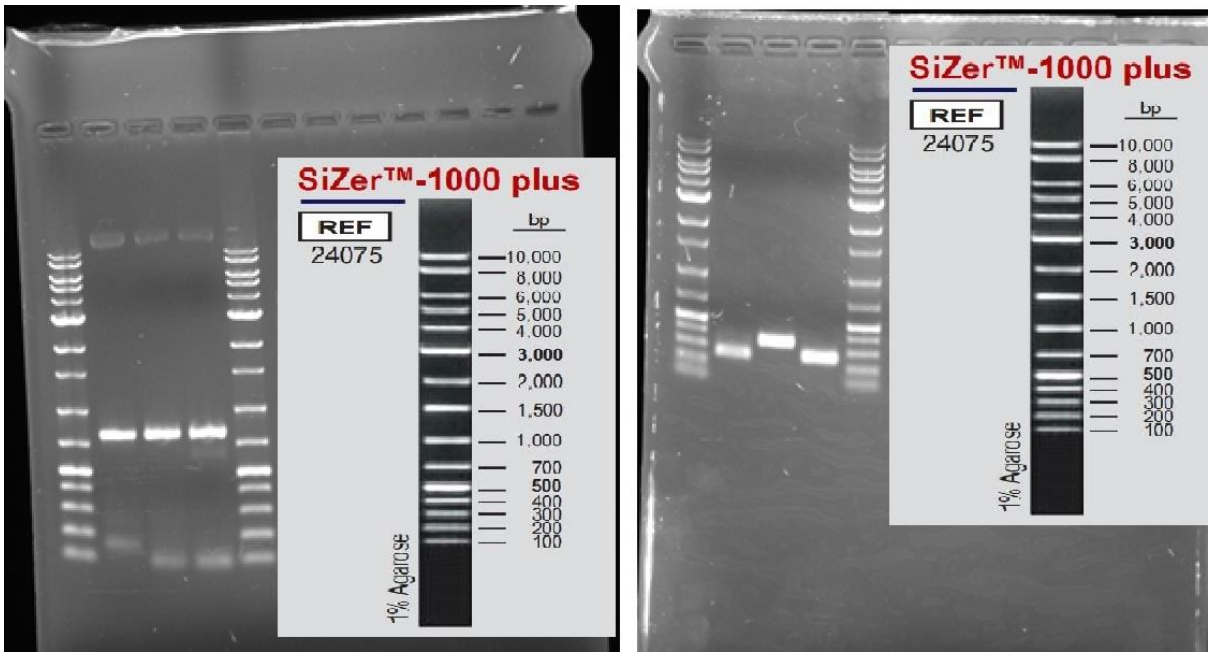


Figure A. 4: Gel electrophoresis images for isolating *mScarlet* and 3 promoter regions. *mScarlet* gene isolated in triplicate around 700 bp in length (Left). Promoter regions from the genes *tolC, soxS,* and *marR* (left to right in right image). Around 204 bp, 300 bp, and 211 bp respectively.

The DNA fragments migrating at the correct predicted position were excised using sterile scalpels and then eluted in sterile buffer at 65°C in elution buffer. DNA concentration was observed using NanoDrop™ 1000 Spectrophotometer (Thermofisher™). The concentrations of the promoters of *tolC, marR* and *soxS* were 13.2 ng/ml, 13.7 ng/ml, and 13.8 ng/ml respectively.

A4 pFru97 digest and isolation

Plasmid 47 (pFru97) was digested using *Hin*dIII restriction enzyme. 34 µL of 50 ng/µL pFru97 (1.8ug) was digested using *Hin*dIII 2 µl.

4µl	10x Buffer R
2µl	HindIII
34µl	pFRU97

This mixture was put on the heating block at 37^{o}C for 90 minutes. I prepared a gel (1% agarose), to purify the pFru97 backbone as in 2.1.5.3. Separated backbone of pFru97 from the gel and was stored in –20C freezer and then purified as in 2.1.5.3. but the minor alterations. These included: instead of using the elution buffer, water (7- 8.5 pH) at 65C was used. This sample was incubated for 3 minutes at 65C. The Gibson assembly required 100ng of DNA per 4.5ul. The volume of water was controlled to yield maximum concentration of DNA in the sample. The concentration of digested pFru97 yielded was 7.2 ng/ml.

A5 Isothermal Assembly

The reaction volume for isothermal assembly was adjusted to 1-part Backbone to 2-parts promoter/mScarlet ratio. This was assembled with Gibson buffer at 50C for 20 minutes. Run according to methods in Gibson et al., 2009.

A6 Making Super-competent Cells

BW25113 grown to OD of 0.4- 0.5 from the OD of 0.05 (LB). Cultures were sun at 5000 rpm for 5 mins at 4^{o}C. The cultures were split up in 50mls, in falcon tubes. Supernatant was discarded, pellets eluted in 2ml of CaCl2 (30 mM). Pellets were combined into a single falcon tube and then CaCl2(30 mM) was added to make the rest of the volume (40 ml). This was spun at 5000 rpm for 5 mins at 4 C. Pellets were washed with 20ml of CaCl2 (30 mM). Spun again at 5000r pm for 5 mins at 4 C. Pellet resuspended in 3ml, 0.1 M CaCl2 +15% Glycerol. Aliquoted in 100 µl samples and frozen.

A7 Transformation

In ice, 10ul of the assembled constructs were added to the competent cells. Incubated 30 minutes. Heat shock, 42^{o}C incubator for 50 secs. 2 mins in ice. Recovery phase, 1 hours in the incubator at 37^{o}C. They were plated on to LB agar plates that contained Kanamycin (4 mg/ml), 100ul on one plate and 900ul on the other.

Colonies were observed for fluorescence daily and colonies showing fluorescence were picked off. Picked off colonies were grown to saturation in LB and kanamycin (40 µg/ml). Saturated cultures were then stored in 15% glycerol at -80^{o}C.

Appendix B: Testing the stability of the plasmids in BW25113 strains

Kanamycin was used as the selective marker to maintain the plasmid in a bacterial culture the due to the plasmid containing the gene *nptII* which gives kanamycin resistance. It needed to be tested whether the plasmid remained in the population long enough to allow for experiments on the strains without kanamycin being constantly present. The bacteria were grown in LB with kanamycin (40 µg/ml) to saturation (approximately $1x10^9$ cfu/ml) at 37°C. The saturated culture was then diluted to a concentration of $1x10^4$ cfu/ml. This was added to two Erlenmeyer flasks in 100 µl quantities, both containing 10 ml of LB broth, one with kanamycin (40 µg/ml). These were incubated at 37°C for 8 hours (approximately 24 generations, assuming 20 minute generation time (Sezonov, Joseleau-Petit, & D'Ari, 2007)). These cultures were then serially diluted and the dilutions of 10^{-5}, 10^{-6}, and 10^{-7} were spot plated as 10 µl drops in triplicate (as in Figure 2.1) on both LB agar plates and LB agar plates with kanamycin (40 µg/ml).

Plates were incubated at 37°C for 18 hours and colonies were counted. Cfu were counted, divided by total dilution factor.

Results indicate there is no significant loss of kanamycin resistance from 8 hours growth (Figure B.1).

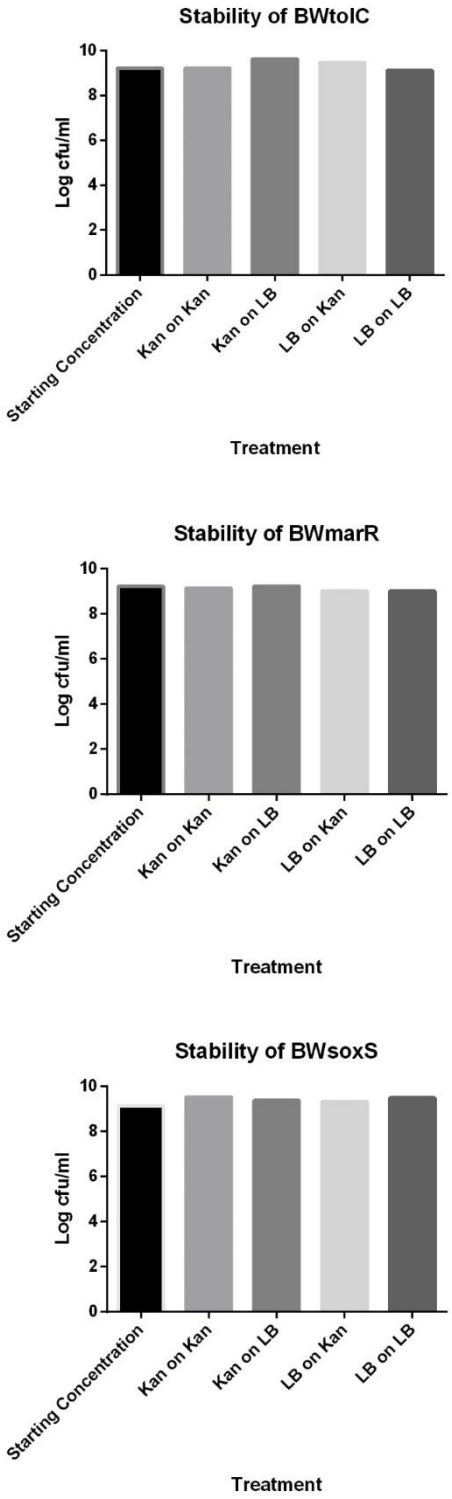


Figure B. 1: Testing stability of the plasmids. Testing stability of plasmid pTolC-mScarlet in BWtolC strain (Top). Testing stability of plasmid pMarR-mScarlet in BWmarR strain (middle). Testing stability of plasmid pSoxS-mScarlet in BWsoxS strain (Bottom).

Appendix C: Testing Growth Rates of BWtolC strain with and without copper ammonium acetate

Fluorescence of individual cells should be measured while in exponential phase of growth in a culture because any fluorescence proteins produced are constantly being divided among daughter cells, and if measurement is done on cells in stationary phase there is a higher chance of the cells having reached a saturation point of fluorescent protein or lower promoter activity in the genes in question (Gefen, Fridman, Ronin, & Balaban, 2014; Kannan, Sams, Maury, & Workman, 2018). Because the introduction of copper ammonium acetate could affect the growth rate of the reporter strains (BWtolC, BWmarR, and BWsoxS), the strains were grown with and without it to test for any differences. To test this for each reporter strain they were grown up as overnight cultures in LB + kan, then serially diluted. 100 µl of $1x10^7$ cfu/ml of the strain being tested was pipetted into an Erlenmeyer flask containing 10 ml of LB. At the same time 100 µl of $1x10^7$ cfu/ml of the strain being tested was pipetted into another Erlenmeyer flask containing 10 ml of LB with copper ammonium acetate (4.73 g/L). Both flasks were then incubated in a 37^{o}C shaker water bath for 180 minutes. After which they were serially diluted, and spot plated on LB + Kan, as shown in Figure 2.1. Colonies were counted and divided by appropriate total dilution factors.

Results indicate a small decrease in the growth rates between Cu- and Cu+. Repetition would be required to establish significance, but the difference appears small (Figure C.1).

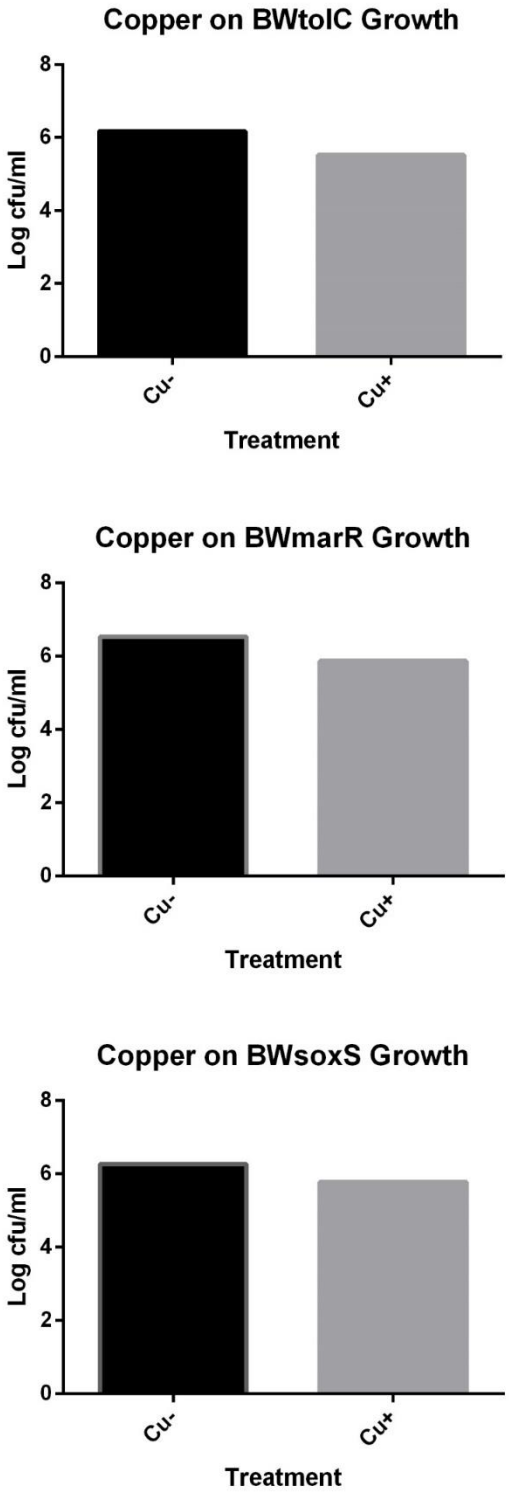


Figure C. 1: Testing the effects of copper ammonium acetate on growth rates of reporter strains. Testing growth rates of reporter strain BWtolC with and without copper ammonium acetate (Top). Testing growth rates of reporter strain BWmarR with and without copper ammonium acetate (Middle). Testing growth rates of reporter strain BWsoxS with and without copper ammonium acetate (Bottom).

Appendix D: Establishing Growth Rates of BWtolC Bright/Dim strain with and without 2,4-D

To determine the effects of 2,4-D on each of the Bright/Dim strains growth rates were established as in section 6.3 but with 2,4-D (1.95 g/L) in the place of copper ammonium acetate and BWtolC Bright/Dim strains in place of BWtolC, BWmarR, and BWsoxS.

Results indicate a no change in the growth rates between 2,4-D exposure and unexposed. Repetition would be required to establish significance, but there appears to be no difference (Figure D.1).

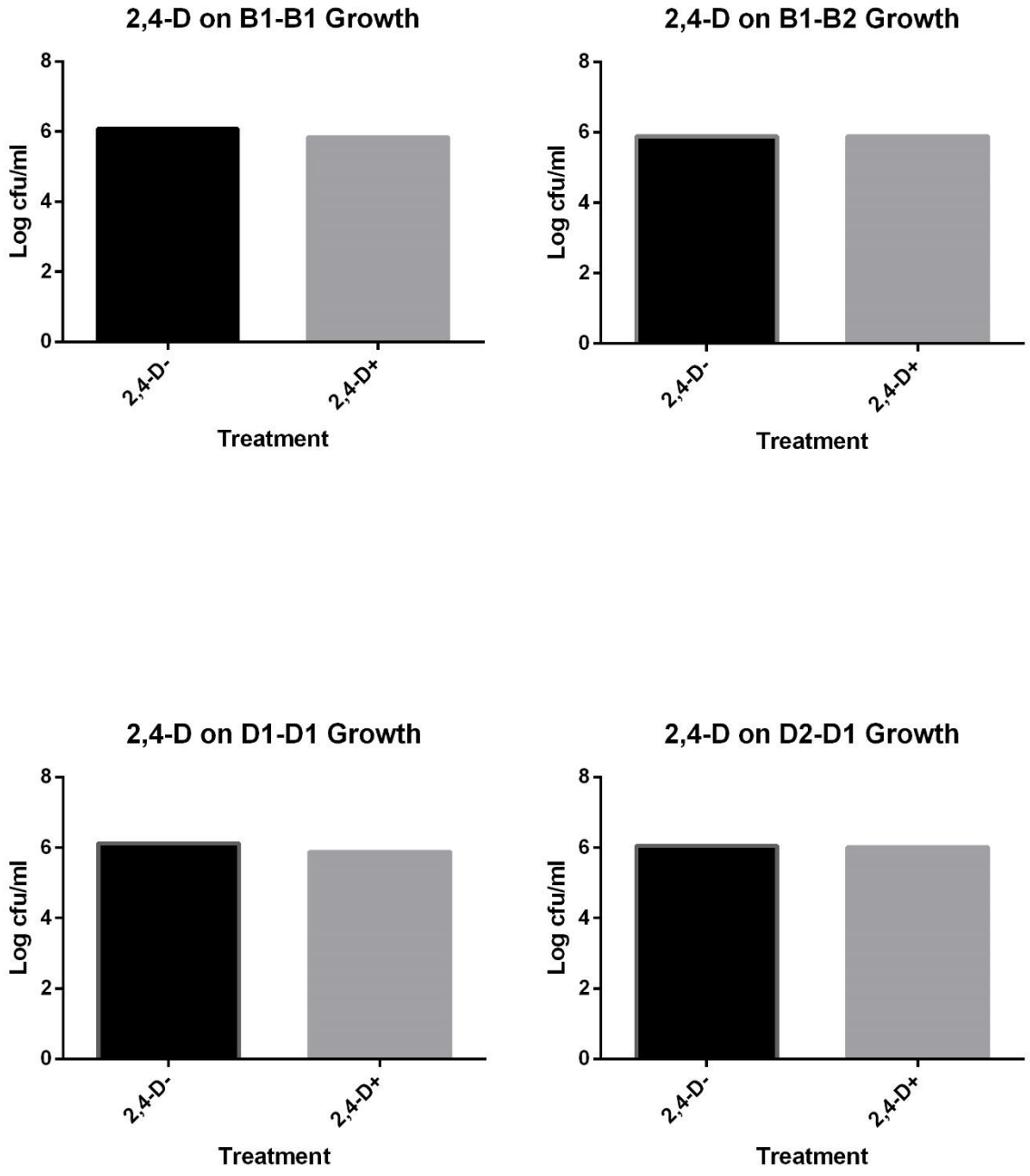


Figure D. 1: Testing the effects of 2,4-D on growth rates of BWtolC bright and dim reporter strains. Testing growth rates of reporter strain BWtolC B1-B1 with and without 2,4-D (Top left). Testing growth rates of reporter strain BWtolC B2-B1 with and without 2,4-D (Top right). Testing growth rates of reporter strain BWtolC D1-D1 with and without 2,4-D (Bottom left). Testing growth rates of reporter strain BWtolC D2-D1 with and without 2,4-D (Bottom Right).